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TUMOR MICROENVIRONMENT: THE PARADOXICAL ACTION OF FIBROBLASTS

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Cover photo
Extended field-TIRF microscopy image (10X), showing the co-culture of turbo-GFP labeled fibroblasts (green) and H2A-mRFP labeled tumor cells (red)

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TUMOR MICROENVIRONMENT: THE PARADOXICAL ACTION OF FIBROBLASTS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“We ourselves feel that what we are doing is just a drop in the ocean.

But the ocean would be less because of that missing drop.”

“Mother Teresa”

Dedicated to

ANKAWA

(My hometown)

“Start by doing what's necessary; then do what's possible;

and suddenly you are doing the impossible.”

“Francis of Assisi”

Thesis Defense

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ABSTRACT

The term tumor refers to an abnormal and pathological tissue characterized by a massive cell growth; it comprises various populations of transformed and malignant cells. These cells cross-communicate with each other and with different types of cells in the surrounding microenvironment. The nature of communication and interactions within the tumor microenvironment (TME) directs the fate of transformed cells via inducing pro- or anti-tumorigenic signals. Consequently, these cells will either succeed or fail to progress into a malignant growth phenotype. In the TME, fibroblasts are considered as one of the major cellular compartments and the primary source of non-cellular elements, including the extracellular matrix (ECM) and soluble factors. It has been shown that tumor cells can recruit fibroblasts to induce growth-stimulatory signals. On the other hand, normal fibroblasts may also act as tumor growth repressors. However, these actions have not been thoroughly addressed. The results of this thesis demonstrate the dual functionality of fibroblasts in the TME. First, we examined the phenomenon, whereby the normal fibroblasts inhibit tumor growth and development. We found that fibroblasts reduced tumor cell proliferation and motility through two sets of signals, the first set involved transmembrane proteins and the ECM. The second set was only effective after induction of the first signal, and included soluble factors secreted upon direct contact of the fibroblasts and tumor cells. Next, we uncovered the signaling pathways that were involved in the process of tumor growth inhibition and fibroblasts activation. We revealed a switch in fibroblasts from tumor suppressive cells to ones characterized by tumor stimulatory functions. Genetic ablation of the *RhoA* gene in fibroblasts significantly reduced tumor cell proliferation and motility *in vitro*, and induced tumor cell clustering in 3D-collagen matrix. Loosing of the suppressive function was accompanied by gaining a tumor inducing ability, since *RhoA* deficient fibroblasts enhanced tumor initiation and development by a small number of PC3 prostate cancer cells injected subcutaneously into immunodeficient mice. In addition, knocking out the *RhoA* gene altered the cytoskeletal organization and reduced *αSMA* expression in fibroblasts. These changes conferred the cells stiffer but less contractile compared to control cells. Furthermore, upon the crosstalk with tumor cells, the *RhoA* deficient fibroblasts overexpressed several pro-inflammatory genes encoding for *IL6*, *IL8*, *CXCL1*, *CXCL5*, and *CCL5*. Such a biochemical and mechanical shift in the fibroblasts reflected their pro-tumorigenic phenotype. Using patient-derived cancer-associated fibroblasts (CAFs). We demonstrated that CAFs rescued tumor cells from apoptosis and could even enhance their growth under cis-platinum treatment. Beside the molecular mechanistic results, this thesis introduces a comprehensive quantitative live-cell imaging tool to investigate tumor cell-fibroblast interactions dynamically, providing the opportunity to measure and observe cellular proliferation, motility, and phenotypic plasticity simultaneously. Taken together, the current thesis uncovers two opposite effects of fibroblasts on tumor growth. These results emphasize the demand for targeting both CAFs and tumor cells to treat and cure cancer patients and may open novel avenues for cancer prevention approaches.

LIST OF SCIENTIFIC PAPERS

I. **Alkasalias T**, Flaberg E, Kashuba V, Alexeyenko A, Pavlova T, Savchenko A, Szekely L, Klein G, and Guven H. Inhibition of tumor cell proliferation and motility by fibroblasts is both contact and soluble factor dependent.
Proc Natl Acad Sci U S A. 2014 Dec 2; 111(48):17188-93

II. Alexeyenko A*, **Alkasalias T***, Pavlova T, Szekely L, Kashuba V, Rundqvist H, Wiklund P, Egevad L, Csermely P, Korcsmaros T, Guven H, and Klein G. Confrontation of fibroblasts with cancer cells in vitro: gene network analysis of transcriptome changes and differential capacity to inhibit tumor growth.
J Exp Clin Cancer Res. 2015 Jun 18; 34:62. * Equal contribution

III. **Alkasalias T**, Alexeyenko A, Hennig K, Danielsson F, Lebbink RJ, Fielden M, Turunen SP, Lehti K, Kashuba V, Madapura HS, Bozoky B, Lundberg E, Balland M, Guvén H, Klein G, Gad AK, and Pavlova T. RhoA knockout fibroblasts lose tumor-inhibitory capacity in vitro and promote tumor growth in vivo.
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IV. **Alkasalias T**, Mohammad M, Moyano Galceran L, Menkens H, Hjerpe E, Carlson J, Arsenian Henriksson M and Lehti K. A quantitative live cell-imaging system reveals fibroblast-mediated alterations in ovarian cancer cell growth, motility and response to platinum treatment.
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LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Liu C, Zhang Y, Lim S, Hosaka K, Yang Y, Pavlova T, **Alkasalias T**, Hartman J, Jensen L, Xing X, Wang X, Lu Y, Nie G, and Cao Y. A Zebrafish Model Discovers a Novel Mechanism of Stromal Fibroblast-Mediated Cancer Metastasis.
Clin Cancer Res. 2017 Aug 15; 23(16):4769-4779

II. Ujvari D, Jakson I, Oldmark C, Attarha S, **Alkasalias T**, Salamon D, Gidlöf S, and Hirschberg AL. Prokineticin 1 is up-regulated by insulin in decidualizing human endometrial stromal cells.
J.*Cell Mol Med. 2017 Aug 7. doi: 10.1111/jcmm.13305 [In press,]*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

αSMA	alpha Smooth Muscle Actin
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1
AC	Adipocyte
CAF	Cancer Associated Fibroblasts
CSC	Cancer Stem Cell
CCL	C-C motif chemokine Ligand
CXCL	C-X-C motif chemokine Ligand
DC	Dendritic Cells
DKK1	Dickkopf-Related Protein 1
EBV	Epstein-Barr Virus
EC	Endothelial cells
ECM	Extracelular Matrix
EDA-A2	Ectodysplasin A2
EMAP-II	Endothelial-Monocyte- Activating Polypeptide II
EMT	Epithelial to Mesenchymal Transition
EP	Epithelial Cell
FAP	Fibroblast Activation Protein
FSP1	Fibroblast Specific Protein 1
GDF15	Growth Differentiation Factor 15
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HGF	Hepatocyte Growth Factor
HPV	Human Papilloma Virus
HTLV	Human T-cell Lymphotropic Virus type 1
IL	Interleukin
IFNγ	Interferon gamma
MDSC	Myeloid Derived Suppressor Cells
MMP	Matrix Metalloproteinase
MYC	Avian Myelocytomatosis Viral Oncogene Homolog
NFκβ	Nuclear Factor kappa-light-chain-enhancer of activated B cells

NK	Natural Killer cell
PC	Pericyte
PDGFR	Platelet Derived Growth Factor Receptor
PGE2	Prostaglandin E2
PTEN	Phosphatase and Tensin homolog
RAS	Family of Retrovirus-Associated DNA Sequences
RB	Retinoblastoma
RhoA	Ras homolog family member A
RSV	Rous Sarcoma Virus
SPP1	Osteopontin
TAM	Tumor Associated Macrophage
TGFβ	Transforming Growth Factor beta
TIMP	Tissue Inhibitors of Metalloproteinase
TNFα	Tumor Necrosis Factor alpha
TNFRSF11B	TNF receptor superfamily member 11b
TME	Tumor Microenvironment
T-reg	Regulatory T cell
u-PA	Urokinase
VEGFA	Vascular Endothelial Growth Factor A

1 INTRODUCTION

1.1 CANCER

Cancer is a worldwide deadly disorder. The term cancer does not refer to a single disease but rather to a large group of malignancies, the majority of which originate from epithelial cells. The cancer incidence varies according to gender, age, geographical distribution, type of cancer, and the level of risk factors[1-3]. Beside genetic susceptibility, the known cancer risk factors such as smoking [4], certain food products [5], alcohol consumption, obesity, low physical activity [6, 7], chemicals, and UV radiation [8] are attributed to the environment. Additionally, chronic inflammations and infectious agents, e.g. Hepatitis B and C viruses [9], Human papilloma virus (HPV), Epstein-Barr virus (EBV) [10], Human T-lymphotropic virus type 1 (HTLV-1), parasitic (*e.g.* the malaria parasite) and bacterial infections (including *Helicobacter pylori*) [11, 12], as well as the tissue specific cell division frequency of noncancerous stem cells [13] have been considered as risk factors for cancer. According to the World Health Organization (WHO), more than 8.5 million deaths were due to cancer in 2015. Therefore, malignancies altogether are considered, as one of the most common causes of mortality and morbidity, and the death frequency is higher in low-income compared with high-income countries [14]. The human cancers are heterogeneous, but share several hallmarks features denoting the cellular-physiology's alterations, including sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genomic instability and mutation, resisting cell death, and deregulating cellular energetics [15].

Altogether, these evidences and observations motivate scientists to study cancer and to explore the fundamental cellular and molecular mechanisms that regulate cancer growth, invasion and the response to treatment.

1.2 THE BIOLOGY OF EPITHELIAL CANCERS

1.2.1 Epithelium

Normal epithelial tissues are composed of polyhedral cells that bind to each other through different cell junctions and active adhesion molecules. They rest on a layer of connective tissues, where an intermediate layer of extracellular matrix (ECM) separates them. The ECM layer is called basement membrane, and is composed of a variety of proteins such as, the

network-forming collagen IV, laminin, nidogen, and proteoglycans. It usually appears as a thick structure under the light microscope. The basement membrane interacts with interstitial matrix of stromal connective tissue. Epithelial cells (EP) cover and line the surfaces and cavities of the body. Ordinarily, they are labile and get renewed as they undergo mitosis, which is a form of cell division. To divide, EP cells require entering the cell cycle, a process that consists of four main phases (G1, S phase, G2, and mitosis, M). The cell undergoes various processes, including DNA replication, cytoskeletal modification, and enzymatic activation during these different phases. Additionally, different circuits or switches control the process of the cell cycle, and thus maintaining the balance and tissue homeostasis [16, 17]. When an error occurs during the cell cycle, several repair mechanisms are available to solve the problem. One of the main players in stopping the cell cycle for repair is the *p53* tumor suppressor, the guardian of the genome [18]. However, as the cell becomes incapable of repairing such defect, a number of intracellular surveillance mechanisms get activated to stop the cell from proceeding further in the cell cycle. Eventually, several multistep programs or signals will be triggered and transduced inside the cell, and these signaling cascades drive the cell into growth arrest or a programmed cell death (apoptosis) [19].

1.2.2 Cancer initiation

When a normal cell loses the control of the proliferation machinery and tolerates the intracellular surveillance mechanisms, it continuously divides, leading to an unordinary cell growth. Such defect in the cellular system is defined as cell transformation, upon which the cell becomes unable to perform normal physiological functions, and instead acquire new genotypic and phenotypic features [20]. The transformation of a normal cell requires genetic and epigenetic alterations, which activate oncogenes and/or inhibit tumor suppressor genes, respectively [21, 22]. Under normal condition, oncogenes (*e.g*. *MYC* and *RAS*), occur as proto-oncogenes, which play important roles in the regulation of cell growth. However, upon activation by means of mutation (gain-of-function), gene amplification, chromosome translocation or epigenetic stimulation, they turn into oncogenes and act as positive regulators of cell growth, which, drive the cell cycle without the requirement of external signals. Different sets of oncogenes are activated in different cancer types, but all cancers harbor oncogenes. The tumor suppressor genes (e.g. *RB* and *p53*), negatively control cell growth, therefore they need to be functionally inactivated in cancer cells. During normal physiological conditions the tumor suppressor genes are involved in the regulation of cell cycle, in DNA repair mechanisms and in induction of apoptosis. The inhibitions of tumor suppressor genes commonly occur by means of mutations (loss-of-function) including

deletions and epigenetic silencing [23-26]. Mutations are dominant in oncogenes and recessive in tumor suppressor genes. Thus, activation of only one copy of an oncogene can be sufficient for a tumor to develop, whereas both copies of a tumor suppressor gene needs to be inactivated [27].

Recently, systems biology approaches have provided tools to cluster cancers according to their tumor suppressor or oncogenic mutational status. However, different cancer types show different genetic and transcriptional signatures; These variations extend over the patients of the same cancer type, as well as over the cell populations in the same cancer patient [28]. The transcriptional alteration can be driven by epigenetic changes, which together with the genetic changes drive tumor initiation process. It is not known which factors determine the cell that exhibits the genetic and epigenetic instability to initiate a tumor. However, it has been proposed that such cells may exhibit stem cell-like properties and are one of the main players in stopping the cell cycle for repair is the p53 tumor suppressor, the guardian of the genome thus called cancer stem cells (CSC) [29]. Also, tumor initiation can be driven via a dedifferentiation process, where the cells lose their specialized features upon epigenetic modifications and regain the progenitor cell functions [30].

1.2.3 Cancer progression

When cell growth remains localized and non-invasive, the tumor is called benign, and rarely causes major medical problems. In contrast, when the transformed cells start to expand and penetrate the neighboring normal environment, they induce malignancies [3]. For transformed cells to maintain their malignant phenotype, they require to sustain their proliferation capacity and eventually become “immortal. One of the key molecules that drive immortalization is the telomere, which indirectly represents the fuel for cell division via protecting the cell from different cell death mechanisms [31]. Therefore, the cancer cells need to maintain telomere length, which mainly occurs via induced telomerase activity [32]. Furthermore, it is necessary for the transformed cells to acquire further genomic and epigenetic changes in order to maintain their malignant phenotype and thus accumulate multiple mutations. The process needs more than four successive genetic and epigenetic alterations in the main cell regulatory circuits; such as the cell cycle, cell death, and phenotypic plasticity [33]. It is difficult to determine the exact number of steps required for cancer development and progression; the main limitation factors for such determination are tumor heterogeneity, time of diagnosis and the availability of early prognostic markers. The

process of cancer progression is a rather a long process, and hence correlates with aging [34]. However, this is not true for childhood cancer, which mostly arise due to hereditary malfunctions or early genetic mutations during development [35].

In this stage of tumorigenesis (tumor progression), the cancer cells tolerate the intra- and inter- surveillance mechanisms, as well as enhance the communication with the surrounding environment in order to invade and metastasize [36].

1.2.4 Cancer invasion and metastasis

The key step in early invasion processes involves the penetration of cancer cells through the basement membrane. The invasive cells gradually remodel the extra-cellular matrix (ECM) and pave the path to facilitate the traveling away from the primary site. During this stage, the malignant cells start to socialize, educate the neighboring cells, and tolerate different cellular surveillance mechanisms. The early invasion, also called microinvasion, usually takes place quietly without leaving any sign behind, thus the host rarely exhibits distinct symptoms. Eventually, the invasive cells succeed in recruiting surrounding stromal cells, which provide important support for invasion [37, 38]. Furthermore, the interactions between cancer and stromal cells boost the invasive property of cancer cells, helping them to intravasate into the blood and/or lymphatic vessels [39]. In few types of cancer such as ovarian cancer, an alternative model of invasion has been described, where tumor cells exfoliate from the primary lesions, float in the ascites and directly reach the peritoneum (first site of metastasis) [40]. Later on, the invasive cancer cells extravasate and establish a new growth on the target organ, this phenomenon called metastasis. Moreover, not all invasive cells manage to initiate a metastatic growth on distant tissues and organs. Interestingly, specific cancer types prefer particular organs to colonize and metastasize. The reason behind such preferential behavior is not well understood [41, 42]. However, the invasive cells encounter new interactions and rely on the recruitment of supportive cancer associated cells at the metastatic site. At the same time, the cancer cells require to tolerate the anti-tumorigenic effect initiated by different type of cells at the pre-metastatic niche. Such action by cancer cells to prime the target site may start before the extravasation step.

Taken together, the resistance to normal microenvironment and the recruitment of cancer associated cells, suggest the possible explanations for a particular niche selection by a given cancer type [43].

1.3 MICROENVIRONMENTAL CONTROL OF TUMOR GROWTH

1.3.1 Overview of tumor microenvironment

The cancer cells with its whole surrounding stromal component are called the tumor microenvironment (TME), which plays an important role during the process of tumorigenesis. It has been clearly shown that tumor development, starting from growth initiation, progression, invasion, and metastasis, is strongly regulated by the surrounding stroma or microenvironment. Therefore, within the last two decades, scientists have shifted their research interest also to the TME in addition to cancer cells themselves. By addressing the biological significance of tumor stroma and its interactions with cancer cells, it has been possible to demonstrate the relevance of such interactions both biologically as well as clinically.

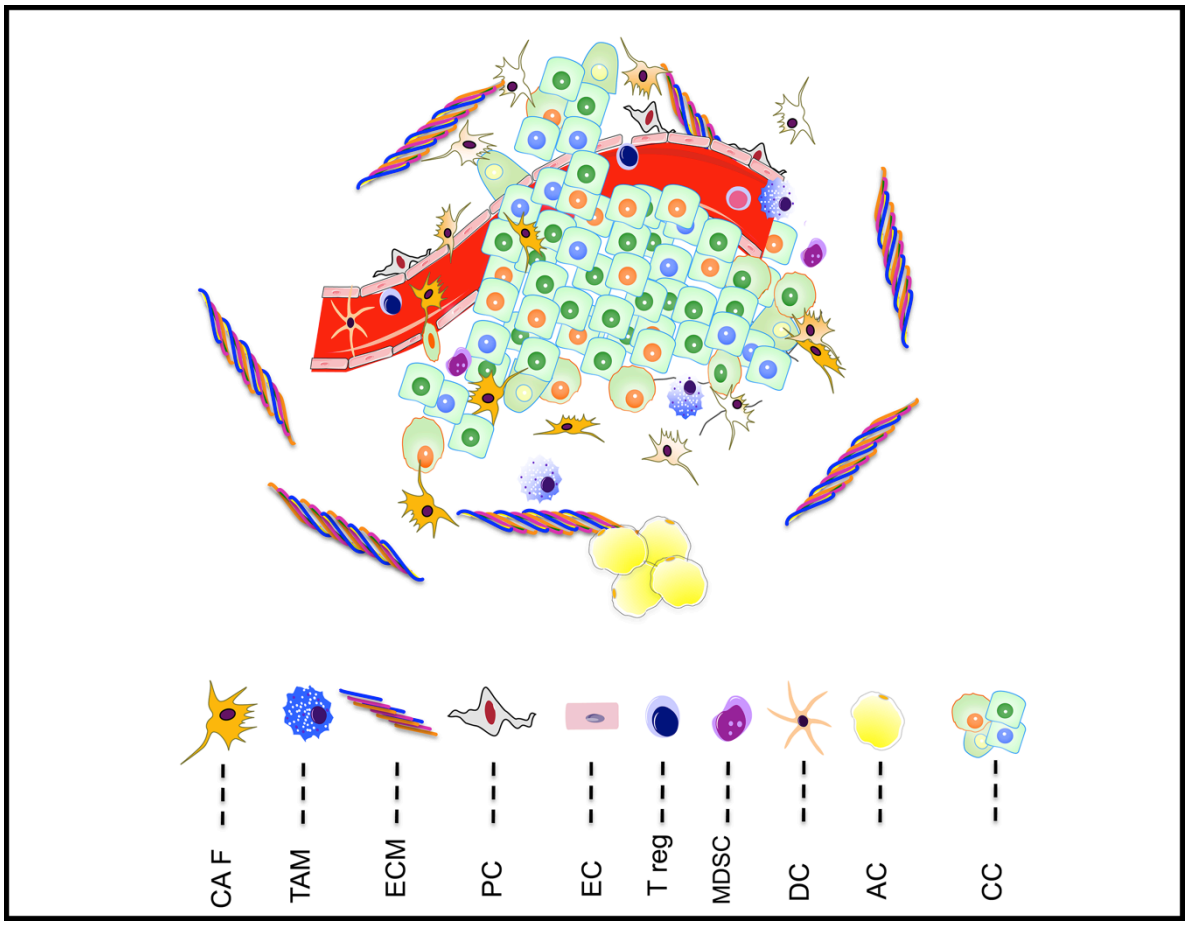

Figure 1. **Illustration of the tumor microenvironment**. Cancer cells with all different types of cellular and non-cellular stromal compartments. (CAF) cancer associated fibroblast, (TAM) tumor associated macrophage, (ECM) extracellular matrix, (PC) pericytes, (EC) endothelial cell, (T reg) regulatory T lymphocyte, (MDSC) myeloid derived suppressor cells, (DC) dendritic cells, (AC) adipocyte, (CC) cancer cells.

The structure of the TME differs according to the type, stage, and location of cancer. The TME can be composed of fibroblasts, endothelial cells, pericytes, macrophages, lymphocytes, and other immune cells as cellular compartments, and ECM as non-cellular compartment (Fig. 1). All these cells interact with cancer cells in multiple ways; the nature of these interactions is dynamic and context dependent. The outcome of tumor-stroma crosstalk is either issuing alliances to help cancer cell invasion, metastasis and resistance against treatment or the negotiation will have a negative impact on cancer cell growth, if the TME will initiate anti-tumorigenic responses [44-46].

1.3.2 Anti-tumorigenic TME

Immune surveillance

The diversity of cellular and non-cellular TME components applies different surveillance mechanisms against cancer development and progression. In the early days, all of these mechanisms were believed to be driven via immune cells [47]. Generalization of the concept might be far from reality. Simply cell transformation and malignancy engages through loss of function rather than gaining a new one. The cancer cells are able to acquire phenotypic plasticity. Therefore, it is not easy for immune cells to recognize the cancer cells as nonself targets [48]. However, the immunocompromised people show higher cancer incidence, and most of these cases, such as EBV-induced immunoblastoma and papilloma related skin and cervical cancers are triggered virally [49].

On the other hand, the contribution of innate immune cells in the process of surveillance is evident. Such cells do not require any antigenic specificity to stimulate their action. Few cells are involved in this phenomenon including macrophages, natural killer cells (NK), and innate lymphoid cells (ILC). [50]. It has been shown that, *CD169*+ve macrophages, localized in the lymph node, act as tumor antigen presenting cells activating *CD8*+ve antitumor T lymphocytes [51]. Moreover, several studies indicate that, both tumor promoting and suppressing macrophages (can) co-exist in the TME [52]. Gillgrass *et al.* have further showed that *IL15* stimulated NK cells are tumor destructive and decrease metastatic lesions of breast cancer in mice [53]. Nevertheless, tumor cells have the ability to modulate and/or inhibit the effect of innate-immune response, thus escaping their killing activity [54, 55]. These findings suggest that immune cell surveillance plays a minor role in the context of host mechanisms to resist tumor [48].

Non-immune surveillance

Humans are considered one of the most cancer resistant organisms, despite the high mutational susceptibility. It's evidenced by the fact that only about one-third of individuals develop tumors. On the other hand, immune cells are inefficient in recognizing and inhibiting cancer cell growth. Altogether, suggests the presence of immune-independent resistance against tumor growth and development, called intercellular surveillance. [56]. Along these lines, Michael Stoker and his colleagues reported one of the earliest investigations on contact dependent interactions between malignant and normal cells. They found that upon contact, the normal cells inhibit the growth of polyoma transformed cancer cells; such phenomenon was called neighbor suppression [57]. Similarly, normal cells have been shown to inhibit the growth of X-irradiated and chemically transformed cells [58]. Interestingly, injecting mouse teratocarcinoma cells into albino mice blastocyte developed fully normal mice, however, their normal organs contained small colonies that persisted during the entire lifespan [59, 60].

Mina Bissell's laboratory reintroduced the concept of Rous sarcoma virus (RSV)-inducing tumors in the chicken [61]. They found that the destruction of the normal tissue architecture upon wound injury, was necessary for tumor development in normal virus-carrying chicken. At the site of damage, the normal cells, due to the healing process lost their inhibitory effect on tumor growth and development. The inhibition of malignancy by normal cells was also recorded upon mixing cancer cells with normal keratinocyte. This effect was maintained through involucrin induced terminal differentiation in transformed cells [62]. Moreover, the detection of the same mutation in the tumor cells and the adjacent normal cells was observed, suggesting that the malignant cells can be enforced in the perspective of phenotypic normalization [63].

The intercellular surveillance phenomenon reflects the concept of "phenotype dominates the genotype". The finding of Partanen *et al*. highlights the relevance of such a concept, where maintaining cell polarity restricts *MYC*-driven cellular transformation, whereas depletion of polarity genes induces the oncogenic effect [64]. On the other hand, the observations of tumor foci found relatively frequent in cancer-free individuals, suggests that intercellular surveillance mechanisms control tumor initiation and progression [65]. Such phenomenon is defined as tumor dormancy, it has been shown that dormant cancer cells can be roused upon chronic inflammation [66]. Recurrence of cancer after ten years from detection of primary tumor [67], is an example for awakening dormant cancer cells at the metastatic site. The chronic inflammation scenario supports the finding of wounding-stimulates RSV-inducing

tumor in chicken [61]. The destruction of microenvironmental architecture can be the key switch for tumor initiation and relapse. These changes will disturb the communication between cancer and normal stromal cells, as well as will disorganize the ECM, allowing the growth and migration of transformed cells [68]. Extracellular matrix, adhesion proteins and cell junctions have been considered as important players to maintain the integrity and tissue architecture. In a three-dimensional microenvironment/experimental setup, human breast cancer cells can re-gain a normal phenotype when cultured with laminin-rich gels [69]. Additionally, reestablishment of E-cadherin (the main structural component of adherence junctions) in cancer cells plays an important role in reverting the transformed cells phenotype [70, 71]. Another example for involvement of ECM and surface molecules in the growth control is β-integrins, which have different sub-types that play an important role in tumorigenesis. In prostate cancer, when the TME architecture is maintained; for instance, upon integrin-laminin polarization, a more differentiated phenotype was sustained. In contrast, breaking down the polarized alignment between laminin and integrins, promotes the malignant phenotype [72]. Interestingly, in naked mole rats, a cancer resistant rodent model, the presence of extraordinary-high molecular weight hyaluronan protects the animal from developing cancer. The only way to induce cellular transformation *in vitro*, is either through knocking down the enzyme hyaluronan synthase 2 (HAS2) or knocking-in the degradation enzyme [73].

The above-mentioned observations support the significance of intercellular surveillance in tumorigenesis. Apart from the TME, there are other surveillance systems clustered under the category of “inter-cellular surveillance”, which have not been highlighted in the present thesis. For a better understanding of tumor resistant behavior of the TME, it is of high demand to perform more comprehensive studies and address such phenomenon systematically.

1.3.3 Pro-tumorigenic effect

Malignancies arise when transformed cells pass the border of inter- and intra- cellular surveillances. Eventually, the structure and functions of the TME will change; instate of initial anti-tumorigenic activity, the TME becomes/switches to protective and supportive for tumor cells [74]. The gaining of supportive function occurs gradually and concurrently with the loss of the inhibitory one. Furthermore, the disturbance of stromal architecture results in accumulation of tissue damage, which in turn initiates different signaling cascades that induce cancer cell proliferation, progression, and invasion [75]. In addition to the inflammation, another examples for accumulation of damage is aging; the

microenvironment undergoes massive changes with age, and correlation between cancer incidence and elderly is well documented [76].

As mentioned, the signatures for early stage carcinogenesis, involves enriched genetic instability and stimulation of proliferation machinery. Subsequently the epithelial cells undergo several morphological changes (atypia), and the abnormal cells produce a small outgrowth called carcinoma *in situ* (CIS)[77]. Such growth will not develop to malignant tumor unless it gets support from the surrounding niche. As transformed cells transduce signals and interact with the stroma through the ECM and surface molecules, they start to grow expansively [78]. Losing of basement membrane integrity is fundamental for achieving such communication. For example, vanishing of Laminin 1, a component of plasma membrane and a key provider of polarity, allowed integrin-mediated direct contact between the transformed cells and interstitial ECM [79]. This contact will induce the tumor cells to secret different factors including matrix metalloproteinases (MMPs), which modulate the ECM and help tumor cells to invade [80]. Tumor cell-ECM interactions induce different signaling cascades in tumor cells, which can be conducted to initiate two actions: first an autocrine effect on cancer cells that can boost their invasive activity, and second a paracrine effect that can enhance the recruitment of variety of cells in the TME. Such cells, obtain an abnormal phenotype to help tumor growth, progression, invasion and metastasis. These cells include fibroblasts, endothelial cells, pericytes, adipocyte, macrophages, and different immune modulatory cells such as, regulatory T-cells (T-regs) and myeloid derived suppressor cells (MDSCs) [46].

Apart from fibroblasts, which the present thesis emphasizes on, the following section will introduce and highlight the contribution of other stromal cells in the process of tumorigenesis.

Endothelial cells (EC)

Endothelial cells are lining the blood vessels, and usually are stable genetically, but acquire phenotypic changes when they are associated with cancer [81]. Recruitment of ECs by cancer cells enhances the process of "angiogenesis", *i.e*. the formation of new blood vessels from the existing ones. This process is highly demanded by tumor cells for two reasons, first; to supply cancer cells with nutrition, oxygen and growth factors, and second; to sustain and enhance cancer cell growth, invasion, and metastasis. The newly formed vasculature has an unstable and disordered structure, as compared to the corresponding normal vessels, and this abnormality is due to the persistently triggered signals and

absences of the normal regulators [82, 83]. Generally, tumor cells and other cells in the TME secrete *VEGF*, which binds to the corresponding receptor on endothelial cells, and as a result, induces a series of signals that break the endothelial cell-cell junctions. The holes between ECs will facilitate the intravasation of cancer cells and also will represent a site for fluid leakage, which in turn enhances the chaotic tumor configuration [84]. Recently Wieland *et al.* revealed that upon activation of the *Notch 1* receptor on endothelial cells via corresponding ligand on cancer cells, the former undergoes different morphological and functional changes, which stimulates tumor cell invasion and metastasis [85]. Also, the crosstalk between cancer cells and endothelial cells may reduce the tumor suppression signaling cascades. It has been shown that, in response to the signals from cancer cells, the ECs downregulate the expression of Slit2, which has a tumor suppressor activity and thus helps in sustaining tumor cell growth [86].

Pericytes (PC)

While ECs are lining the lumen of blood vessels, PCs locate on the other side of the basement membrane, which forms the main adhesion surface to both of these cell types [87]. The PCs establish a physical contact with endothelial cells and support the overall vessel's structure, whereas, upon recruitment by tumor cells, the contact become loose and weak [88]. The recruitment of PC is associated with different signaling pathways such as *TGFβ*, *PDGF*, *Notch* and angiopoietin. Most likely the PC-tumor cell interaction is beyond the two dimensional relationship; this means that other stromal cells can influence the PC-tumor cell interaction, for *e.g*. ECs secret *PDGF* that bind to corresponding receptor in PCs and *PDGF*-activating PCs support tumor cell growth and invasion [89]. Tumor associated PCs are characterized by expression of different markers such as *α-SMA, PDGF* receptor α and β, desmin and others [90]. According to the type and stage of cancer the PCs may show different responses; for example, the effect of depleting PCs in the early stages of carcinogenesis has been found to be negative on cancer cell growth. In contrast, when the depletion was maintained at advanced stages, the cancer cell growth and metastatic rate were induced significantly [91]. In addition, the number of PCs on vasculature can affect cancer cell growth and invasion, since the coverage density of PCs was proportionally related to low invasive property of cancer cells in a prostate cancer model [92].

Adipocytes

The knowledge about adipocytes as inflammatory cells has recently been emerging. Previously, these cells were believed to mainly represent storages for lipid and energy [93].

These cells are now known to produce different cytokines and adipokines, such as *IL-1β, TNFα, CCl2,* and *IL6*, changing the inflammatory properties of the TME and the recruitment of various immune cells [94]. However, such switch in the nature of microenvironment resembles the condition of chronic inflammation, which shifts the TME to be more pro-tumorigenic [95, 96]. In a mouse model, macrophage infiltration was decreased upon obesity-induced inflammation [97]. On the other hand, upon recruitment by tumor cells, adipocytes can dedifferentiate back to fibroblast like cell, releasing free fatty acids, which can be utilized by cancer cells as source of energy to sustain their growth and invasive phenotype [98]. In a breast cancer models, the cancer associated adipocytes induce tumor cell growth and aggressiveness [99]. Additionally, in mice lacking the *Stromelysin-3* (*MMP3*) gene, the adipocytes decreased the initial cancer cell survival and invasion into the surrounding connective tissue [100].

Immune cells

Among the variety of immune cells in the TME, macrophages constitute an abundant sub-type [101]. The polarization of macrophages by tumor cells occurs through a variety of ligands, which modulate macrophage functions. They show a wide range of plasticity in response to cancer growth and progression. Broadly, macrophages can be categorized into two sub-types; the phenotype, which shows an anti-tumorigenic effect, called type 1 macrophages (M1), whereas type 2 macrophages (M2) represent the tumor stimulatory phenotype [102]. Generally, pro-inflammatory ligands such as *TNFα, IFNγ*, lipopoly-saccharide and *GM-CSF* stimulate M1 macrophages [103, 104]. M2 macrophages get stimulation through *TGFβ, IL10, IL4, IL13* and glucocorticoids [103, 105].

In response to stimulation, the M1 macrophages induce the pro-inflammatory signature of the TME via secreting different cytokines including *IL1β, IL6, and TNFα*. In contrast, the M2 subtype secretes more anti-inflammatory ligands such as *IL10, TGFβ*, prostaglandin E2, and *IL1* receptor antagonist. The M2 subtype also induces the angiogenic signals and MMP expression by stromal cells [106]. It has been shown in ovarian cancer patients that the high M2 density correlates with bad prognosis [107], while the high M1/M2 ration correlate with a better survival [108]. Additionally, increased numbers of M2 macrophages were recorded in prostate cancer tissue as compared to intraepithelial neoplasm and normal prostate [109].

In addition to TAM, there are other immune cells recruited into the TME. Myeloid derived suppressor cells (MDSCs) are recruited from the bone marrow into the TME. These cells normally suppress the immune activity [110], and the tumor associated MDSCs have been

reported to suppress the anti-tumor activity of immune cells in different cancers. In pancreatic cancer mouse model, the recruitment of MDSCs inhibits cytotoxic T cells *in vitro* and enhances tumor growth *in vivo* [111]. In recent study, the Hippo-YAP pathways showed to promote the recruitment of MDSCs, which were essential for prostate cancer growth and progression [112]

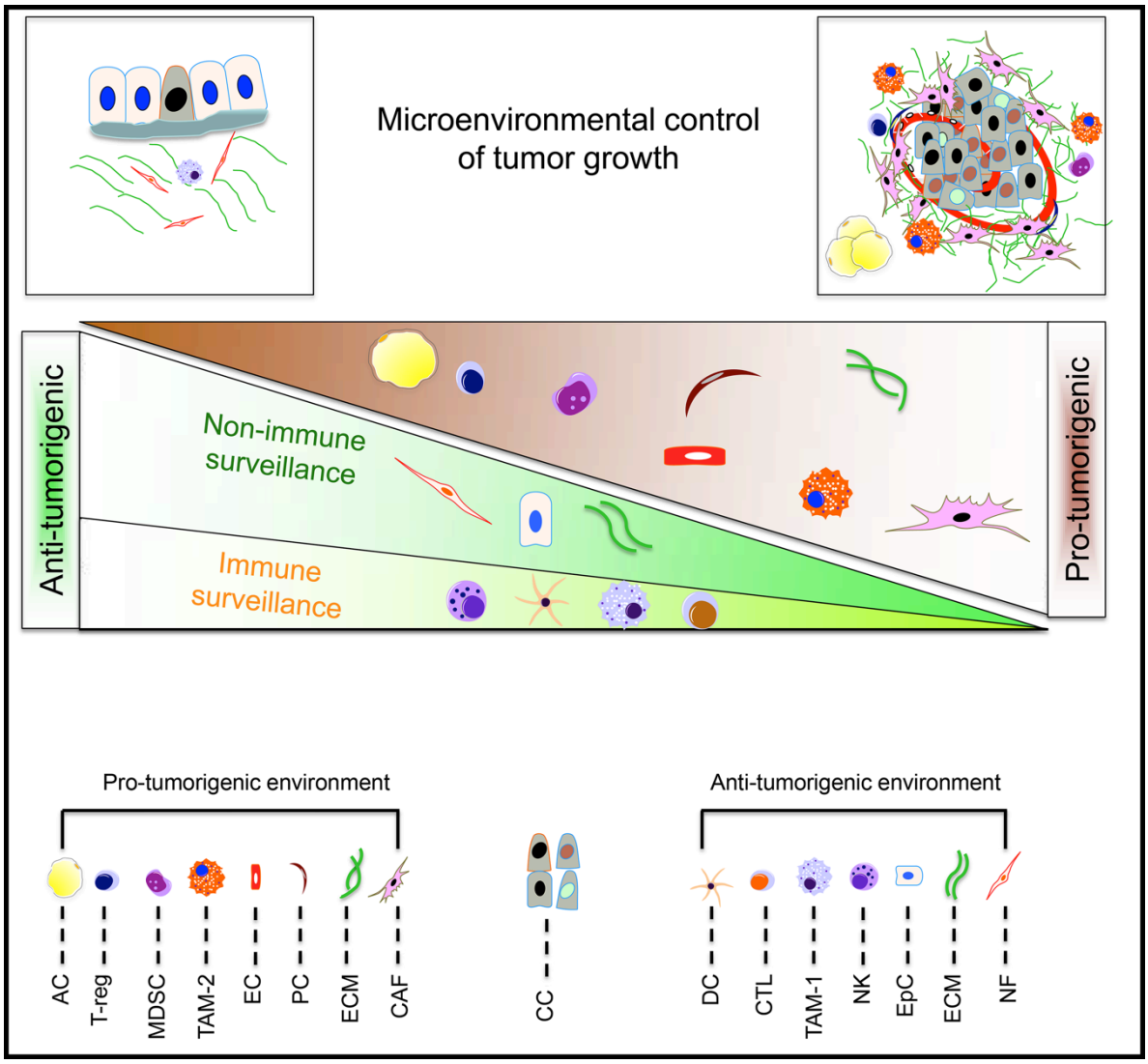

Figure 2. **Microenvironmental control of tumor growth**. The TME can either act as tumor growth suppressor and shows anti-tumorigenic effect, or act as tumor growth stimulator and induce pro-tumorigenic effect. Different TME compartments are involved in driving such effects. AC (adipocyte), T-reg (regulatory T cell), MDSC (myeloid derived suppressor cell), TAM-2 (tumor associated macrophage-type 2), EC (endothelial cell), PC (pericyte), ECM (extra cellular matrix), CAF (cancer associated fibroblasts), NF (normal fibroblast), DC (dendritic cell), CTL (cytotoxic T lymphocyte), NK (natural killer cell), EpC (epithelial cell), TAM-1 (tumor associated macrophage-type 1), CC (cancer cell).

The regulatory T-lymphocytes (T-regs), are another example of pro-tumorigenic immune cells recruited into the TME. The infiltration of T-regs into the tumor has been correlated

with poor patient survival. However, recent observations indicate the existence of different sub-types of T-regs reflecting their heterogeneous and context dependent responses (in different cancer patients) (for review [113]).

Collectively, the microenvironmental control can drive the process of tumorigenesis forward or backwards (Fig. 2). All cellular and acellular components of the TME interact with cancer cells and with each other to orchestrate this process.

1.4 FIBROBLASTS

1.4.1 Overview of the structure and origin of fibroblasts

Fibroblasts constitute the most abundant cell types in the stroma. They produce and reorganize various ECM proteins, supporting the architecture of tissues and organs. Such organization provides tissues and organs with the appropriate environmental condition to perform their functions efficiently. Additionally, fibroblasts interact with the neighboring epithelial, endothelial and immune cells, as they secrete and respond to different signaling molecule including cytokines, chemokines and growth factors. Consequently, they play a vital role during the process of tissue development, repair, and homeostasis [114-116]. The current knowledge has shifted fibroblasts from being rather generic to more tissue specific and context dependent cells. A comprehensive study by Rinn *et al.* showed that human fibroblasts exhibit transcriptional programs according to their anatomical site of origin. A total of 47 primary fibroblasts, isolated from 43 different anatomical sites were included in the study. The mutual genetic expression pattern allowed the clustering of fibroblasts into three anatomical location-based categories; first, anterior and posterior, second, proximal versus distal, and third, dermal against non-dermal sites of the body [117]. Later the same research group found that these genetic variations in fibroblasts are controlled and maintained by epigenetic modifications [118]. Altogether, these results highlight the plastic nature of fibroblasts.

Fibroblasts are quiescent cells during normal physiological conditions, whereas stress or injury stimulates them at the sites of tissue damage. This is the situation during inflammation, wound healing, and fibrosis-induced diseases. What is unclear is whether the recruited fibroblasts originate mainly from local quiescent fibroblasts or from other types of cells by transdifferentiation. As shown in Figure 3, the tissue injury or inflammation-associated fibroblasts can originate from at least four different cell types [114, 119]. These include local tissue resident fibroblasts. However, the low proliferative rate of tissue-resident fibroblasts challenges the model of local fibroblast activation. Fibroblasts can also arise from epithelial

cells through a process called epithelial-to-mesenchymal transition (EMT), which can occur during inflammation, cancer, rheumatic arthritis, and other pathological conditions [120]. During this process, the epithelial cell loses the epithelial cell junctions and polarity, coincident with cytoskeletal reorganization and morphological change [121]. Mesenchymal and endothelial progenitor cells could also be considered as the precursors for the accumulation of fibroblast like cells [122]. In a rabbit ischemic model, angioblasts could be recruited from the blood stream into the sites of vasculogenesis [123]. It has been shown that cells of mesenchymal phenotype are circulating in the blood; interestingly, such circulating cells share similar characteristics with the fibroblasts that accumulate in the joints of patients with rheumatic arthritis [124].

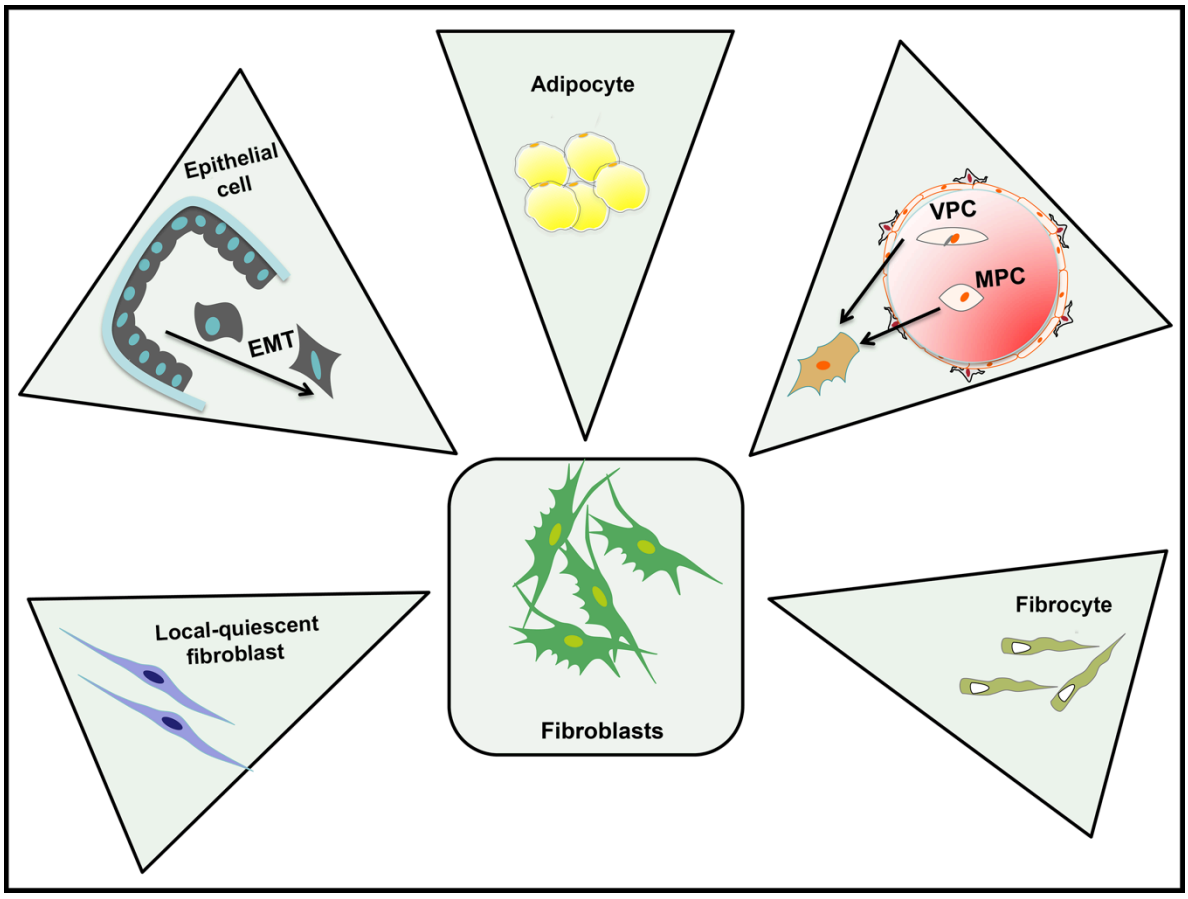

Figure 3. Fibroblasts differentiation process. Upon stimulation fibroblast arises from different type of cells. MPC (mesenchymal progenitor cell), VPC (vasculature progenitor cell).

Another putative source for disease-related accumulation of fibroblasts are fibrocytes, which constitute less than 0.5% of non-erythrocytic cells in the blood. They can induce tissue remodeling upon entry into the site of injury. Fibrocytes are spindle-shaped adherent cells and arise from a sub-type of blood circulating monocytes. They differentiate into fibroblasts in response to TGFβ and other cytokines [125, 126]. Since both fibroblasts and adipocytes are

of the same mesenchymal lineage, it has been suggested that, during carcinogenesis, the adipocyte represent the source of active fibroblasts [127]. Adipose tissue mesenchymal cells can convert to fibroblast like cells that induce the growth of human pancreatic cancer cells in BALB/cAJcl-nu/nu mice [128].

1.4.2 Functions of fibroblasts

The most important functions of fibroblasts include the ECM production, degradation, and interactions. Therefore, they are considered as essential elements driving matrix homeostasis [129]. Similarly to the type of fibroblasts, the configuration of ECM varies according to the localization and type of tissue. Such diversity provides a framework for the tissue specific-residential cells to navigate through the ECM [130, 131]. Examples for the ECM proteins produced by fibroblasts are collagens (e.g. type I, III and V), proteoglycans, fibrin, fibronectins, glycosaminoglycans, and other ECM fibrils, which configure into a three dimensional network and generate an osmotic-active scaffolds [129, 132, 133]. Fibroblasts also participate in the formation of basement membranes via synthesizing and secreting laminin and collagen IV [134]. Additionally, upon stimulation during injury or pathological conditions, the activated fibroblasts interact with ECM through different adhesion molecules and signaling receptors. As a result, and according to the type of interactions and stimulation, the fibroblasts synthesize and degrade particular ECM molecule [135]. Fibroblasts express different surface adhesive molecules such as integrins, sydecans and cadherins, which represent the mediators for fibroblasts-ECM interactions. Such cell-ECM interaction engages a range of signaling cascades, which influence cellular proliferation status, enhance or inhibit the pro- and anti-inflammatory responses, directing apoptotic versus survival signals, and inducing the secretion of different soluble and growth factors [136, 137].

Fibroblasts participate in ECM remodeling via expression of different matrix-degrading proteases (including their inhibitors), which are enzymes essential to tissue maintenance and repair. Several families of these enzymes are known, including *MMPs*, cathepsins, urokinase-plasminogen system proteins tissue inhibitors of metalloproteinases (*TIMPs*), and aggrecanases [138, 139]. Generally, fibroblasts produce different types of *MMPs* upon stimulation via various pro-inflammatory cytokines and growth factors, such as *IL1α* and *β,* fibroblast growth factor (*FGF*), *PDGF* and others [140, 141].

In addition to the ECM synthesis and remodeling, fibroblasts recruit immune cells to the site of damage by producing a wide repertoire of inflammatory mediators, or expressing Toll-like receptors (TLRs). Additionally, they can sensitize bacterial lipopolysaccharide (LPS) through

secreting range of chemokines, which in turn induce the recruitment of inflammatory cells [142, 143].

1.5 FIBROBLASTS: AN ASPECT OF NEIGHBOR SUPPRESSION

The neighbor suppression is a phenomenon defined as the ability of particular normal cells to inhibit the growth of adjacent abnormal or transformed cells by direct or indirect contact. Such phenomenon represents a vital form of intercellular surveillance [144]. Michael Stoker and co-workers discovered that mouse fibroblasts, upon contact, inhibit polyoma virus-transformed cells *in vitro* [57]. Surprisingly, Kirk *et al*. showed that the inhibition of tumor cell growth by normal lung fibroblasts is contact independent, but require the presence of the fibroblast secretome [145]. Other studies demonstrated that the paracrine inhibitory effect of *TGFβ, TNFα, and IL6* against tumor cells is triggered upon mixing tumor cells and fibroblasts in a co-culture system [146-148]. The inhibitory effect of fibroblasts against their transformed derivatives can be mediated through the gap junction intervening the communications between these two types of cells [149, 150]. However, conflicting results have been reported in a different study, where the inhibitory effect was shown to be independent of gap junction [151], but depend on the type of fibroblasts and transforming oncogene [151, 152].

On the other hand, about four decades after his first discovery, Stoker and his colleagues re-introduced the concept of "neighbor suppression" based on results with the suppressor and non-suppressor mouse fibroblasts against SV40 transformed derivatives. They found that the inhibition was independent of gap junction or apoptosis, but it was driven through the cell cycle arrest in G2/M phase [153]. Furthermore, Flaberg *et al.* from our group, showed by using a high throughput proliferation assay, that mouse fibroblasts are capable of inhibiting both; the mouse transformed fibroblasts and human tumor cells, suggesting that the inhibitory effect could predominate across species [154]. The study was further extended to investigate the impact of 107 primary human fibroblasts isolated from pediatric and adult patients, obtained from different tissues including skin, mastectomy-resected tissue, inguinal-hernia, nasal polyps, and prostate. However, to compare the suppression efficiency, the fibroblasts were clustered into two groups, one represented the internal organ fibroblasts and the other as skin fibroblasts. The effect was investigated against six different human cancer cell lines; three from prostate, two from lung and an EBV transformed lymphoblastoid cells. Interestingly, fibroblasts inhibited the cancer cell proliferation, and the range of inhibition varied according to the fibroblastic-site of origin and donor's age.

Skin and pediatric fibroblasts were more effective suppressers of tumor cell proliferation, as compared to the internal and adult fibroblasts [154]. Moreover, in a separate study, the inhibition of tumor cell proliferation and activity depended on the architecture of fibroblast monolayer in the co-culture. The confluency of fibroblast monolayer affected the proliferation score of cancer cells; the more confluent the fibroblast layer was, the more inhibitory the fibroblasts were [155]. Normal dermal fibroblasts have also been found to inhibit the onset of melanoma tumor in mouse model; fibroblasts enhanced cell cycle arrest in melanoma cells, which showed reduced p16 and cyclin D1 levels thus low proliferation rate [156].

Several questions can be addressed about the potential relevance of neighbor suppression, in specific during cancer initiation and dormancy. More comprehensive studies are required to investigate the molecular mechanism behind this phenomenon and to identify the possible links between cancer inhibiting and cancer promoting phenotypes.

1.6 QUIESCENT, ACTIVATED, AND CANCER ASSOCIATED FIBROBLASTS (CAF)

Fibroblasts, at the physiological conditions, are generally localized in the interstitial stromal spaces between the parenchymal tissues [157]. They have a quiescent phenotype, which from a molecular perspective still remains incompletely characterized. However, they are known to be susceptible to a various stimuli, upon which their physiological status changes and they become activated [158]. The activated fibroblasts present inexhaustible protein synthetic activity and contractile functions, which are fundamental during the formation of new connective tissue and throughout the wound healing process [159].

Upon activation, the shape of fibroblast changes from fusiform, bland and elongated to a wide-cruciform structure, also called myofibroblasts (Fig. 4). The activated cells express several markers that can be distinguishable *in vivo* and *in vitro*, such as *αSMA, PDFGRβ*, and fibroblast activation protein (*FAP*) [160]. In contrast, absolute markers for the identification of quiescent fibroblasts are still under debate. The one that is routinely used called fibroblast specific protein 1 (*FSP1*), also called S100A4. However, it is expressed also in other cells such as macrophages and few cancer cells [161, 162]. When compared to quiescent fibroblasts, the activated cells are more migratory and vulnerable for epigenetic modifications, which allow serving as precursors for different cell types. Eventually, they enhance their proliferation machinery, ECM production and altered secretome [163-165].

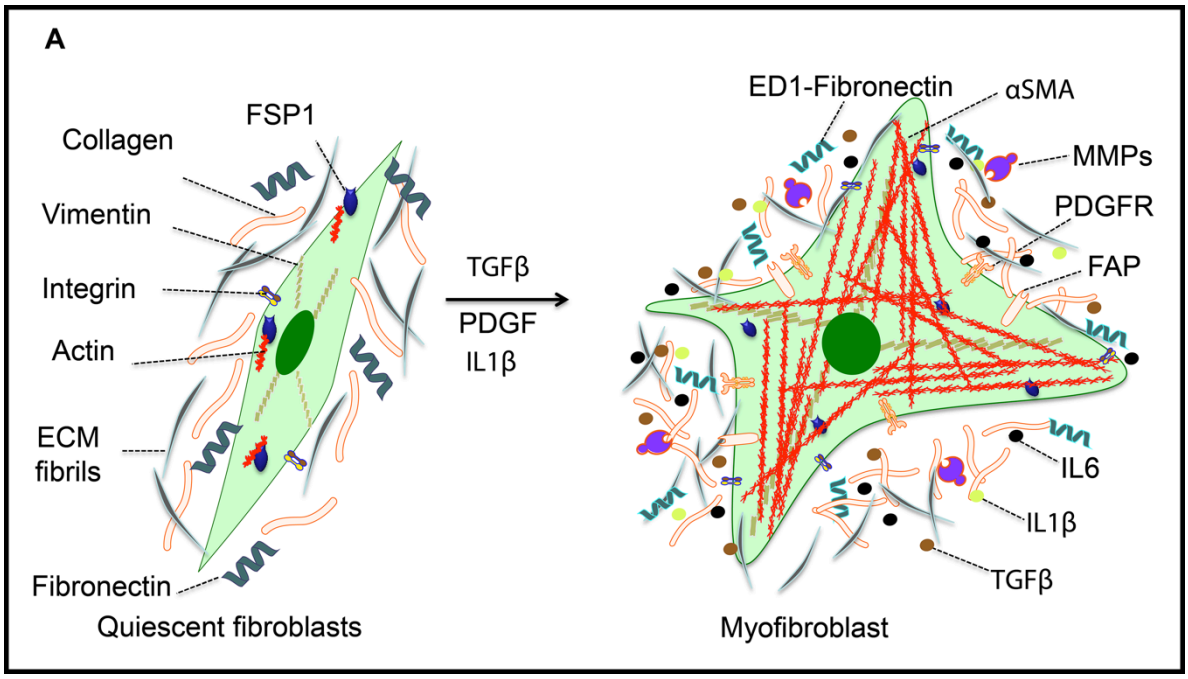

Figure 4. Fibroblast activation process (classical theory). Quiescent fibroblasts respond to stimuli such as TGFβ, PDGF, and IL1β, and thus exhibit morphological changes and express range of markers such as SMA, FAP, PDGFR and secret various proteins including MMPs, interleukins and growth factors.

Recently, the notion of fibroblast activation process has been challenged, suggesting the existence of two activated phenotypes. The first is reversible, which is indicated during wound healing process; the fibroblasts are morphologically flattened and express *αSMA* and Vimentin (Fig 5). Also they acquire contractile properties, induce cytoskeletal rearrangements, and enhance ECM production and remodeling. Such features are accompanied with moderate, but adequate, secretory functions to fuel the microenvironment and sustain the proliferation and migration machineries. As the repair process is accomplished, the activated fibroblasts undergo programed cell death or experience epigenetic reprogramming [132, 166]. It has been shown that myofibroblasts exhibit a transient activated phenotype and can be de-differentiated to quiescent form in the presence of IL-10 [167].

The second sub-type of activated fibroblasts is irreversible, where the fibroblasts are continuously exposed to the stimuli. Such phenotype represents the CAFs, which accordingly obtain unique features assembled as excessive and specific secretory and ECM remodeling phenotypes. Also, they acquire an autocrine signaling ability and a greater proliferative efficiency [166, 168]. Generally, the activated fibroblasts require epigenetic modification in order to convert into pro-invasive CAFs. It has been shown that LIF (leukemia inhibitory factor) induces an epigenetic switch in the fibroblasts resulted in

continuous activation and obtaining cancer-associated phenotype [169]. The special immunomodulatory functions of CAFs is represented by massive production of cytokines and chemokines, including *PDGF, VEGFA, PGE2, IL-6, TNF, NF-κB, IL-8, HGF*, and *CXCL12*, [170-174]. Moreover, their specific ECM remodeling ability is attributed to the production of certain MMPs, such as *MMP1, MMP2, MMP3, MMP9, MMP13,* and *TIMPs* [175-178]. CAFs can be identified, both *in vivo* and *in vitro*, through a panel of markers such as, *PDGFR α and β, αSMA, FAP,* and *FSP1* [179-181]. Since these proteins can be expressed by different cells, other than CAFs, therefore, it is recommended to use more than two - three of them simultaneously, along with specific epithelial or tumor markers, as a control, to identify them precisely. Furthermore, different studies revealed different CAF signatures, for example, analyzing more than 2500 proteins using the Protein Atlas database revealed twelve new CAF markers (*ARHGAP26, ARHGAP31, AZI2, BHLHE40, DLG1, EGLN1, ITCH, PKM2, PLOD2, RAB31, ROCK2,* and *RNF19A*) [182]. The signature was identified to represent CAFs in five different cancers (lung, colorectal, breast, basal cell and squamous cell carcinoma). In a colon cancer study, using quantitative proteomic analysis a new CAFs signature was identified, as assembled by four markers (*CDH11, FSTL1, LTBP2,* and *OLFML3*) [183]. Such observations indicate that CAFs are highly heterogeneous cell populations, revealing a definite expression patterns depending on the type of cancer or even within patients of the same cancer type. It has been shown that in different cancers, such as colon, esophageal squamous cell, non-small cell lung, and breast cancer, different patients (diagnosed with the same cancer type) exhibited different fibroblastic-gene signatures. Moreover, such variety could be used as prognostic factors since it clustered the patients into high and low risk groups [184-187].

Altogether, the two-step fibroblast activation model, differentiating the normal active fibroblasts from the CAFs, further supports the concept of fibroblast's dual behavior against tumor cell growth and development. Investigating the exact molecular mechanisms, which direct the action of each phenotype, thus defining them autonomously, may significantly drive our knowledge toward the possible cancer treatment and prevention strategies.

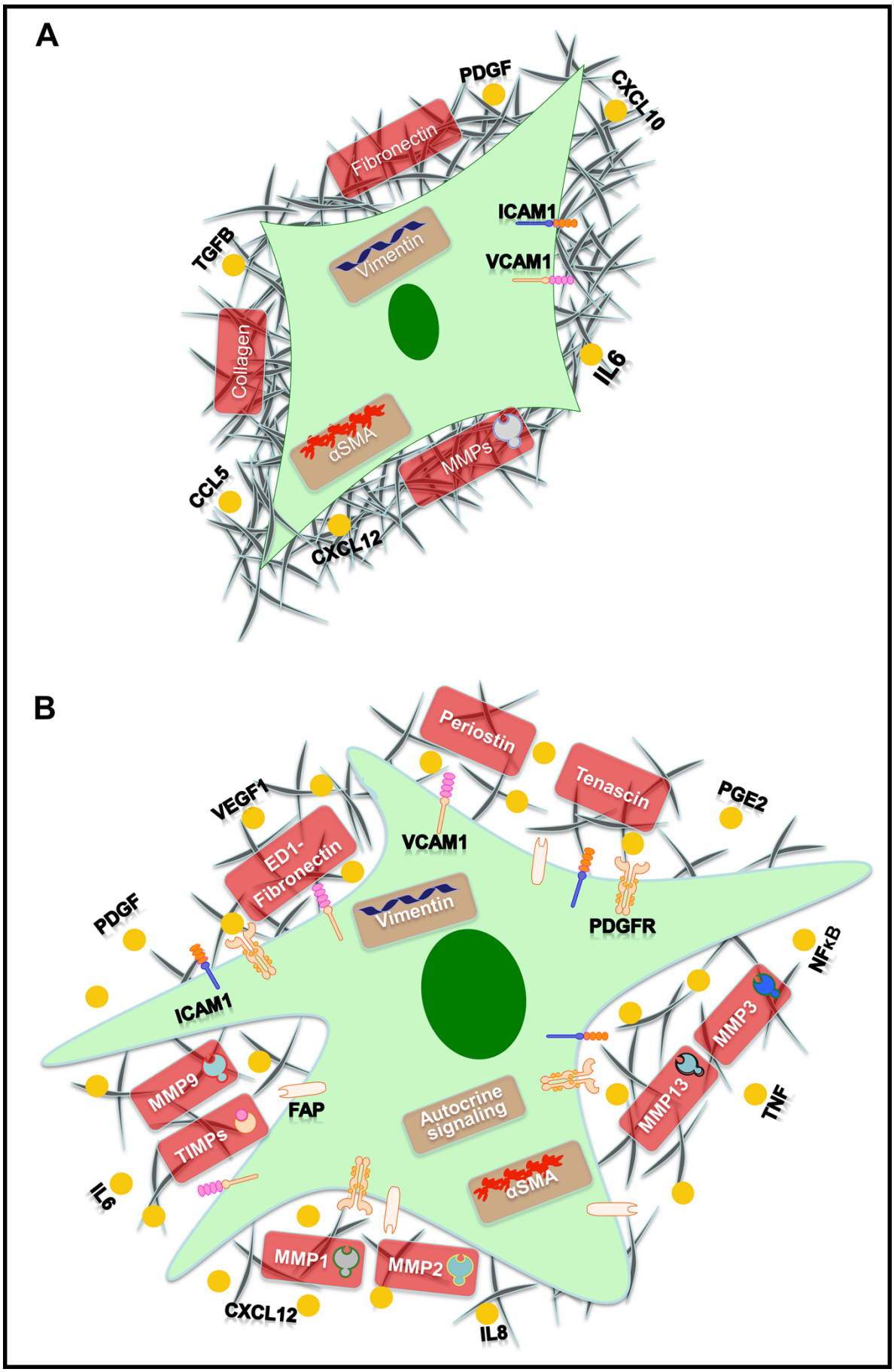

Figure 5. The two-stage model of active fibroblasts. A) Normal active fibroblast (reversible activation), B) Cancer associated fibroblast (irreversible activation). The CAFs acquire specific and effective secretory properties and also exhibit unique ECM remodeling properties.

1.7 CANCER ASSOCIATED FIBROBLASTS: AN ASPECT OF CARCINOGENESIS

1.7.1 The impact of CAFs on cancer initiation

The switch of normal fibroblasts into CAFs is one of the most fundamental steps in tumor development. Due to the difficulty in defining the threshold of cancer onset, the impact of fibroblasts on tumor initiation process is debatable. The concept of "the egg and chicken" is applicable; who comes first? Transformed cell recruit fibroblast or the activated fibroblast induces epithelial cell transformation and malignancy? Observations, which highlight the merit of each strategy over the other, are available. The majority of these studies have been done in mice or *in vitro*. One of the investigations revealed that CAFs isolated from prostate cancer patients induced epithelial cell transformation and immortalization, as well as, shifted the non-tumorigenic feature of the epithelial cell line into highly tumorigenic one [188]. Different experimental models, such as gene modification, overexpression and deletion contributed to demonstrating the role of fibroblasts in tumor development processes. One of the studies showed that Wnt1 overexpressed fibroblasts could transform mammary epithelial cells isolated from C57BL/6 mice [189]. A number of observations suggested the contribution of *TGFβ* signaling in the process of cancer initiation. A study performed in mice, an irradiated microenvironment decreased cancer latency and boosted the development of aggressive mammary tumor upon *p53*-null epithelial cells transplantation in mice. This effect was mediated through TGFβ; genetically knocking down *TGFβ* abolished the effect on latency [190]. Furthermore, knocking-out *TGFβ* receptor II in *FSP-1*-positive fibroblasts promoted prostate intraepithelial neoplasia and fore-stomach squamous cell carcinoma [191]. Whereas, in *FSP1* (*S100A4*)-null mouse model, the mice showed significantly delayed and decreased tumor initiation rate upon injecting highly metastatic mouse mammary carcinoma cells, while when the cancer cells were co-injected with fibroblasts expressing *FSP1*, they partially enhanced tumor development process [192].

Trimboli *et al.* showed that *PTEN* inactivation in fibroblasts significantly enhanced the malignant transformation, initiation, and growth of mammary adenocarcinoma in mice. They also observed immune cells infiltration and substantial increased ECM remodeling, and interestingly the transcriptomic analysis of *PTEN*-inactivated fibroblasts showed a high correlation with breast CAFs in human patients [193]. An elegant study showed that senescence drives the fibroblasts to induce tumor growth, where osteopontin-producing

senescent fibroblasts enhanced the pre-neoplastic growth of epithelial cells in mice and in vitro; the effect of osteopontin was mediated through the activation *MAPK* pathway.

Moreover, senescent fibroblasts promote tumorigenesis through expressing IL6 and recruiting immunosuppressive signals [194-196]. A recent study highlights the role of CAFs in initiating ovarian cancer growth *in vivo* and inducing sphere formation *in vitro*. This fibroblast function was through enhanced expression of *FGF4*, which binds to *FGFR2* on tumor cells and this signaling was essential to boost the growth and proliferation of ovarian cancer cells [197].

1.7.2 The impact of fibroblasts on cancer progression

Several observations and studies highlight the important role of CAFs in the process of tumor growth and progression, indicating a significant impact on tumor identification and suggesting new treatment strategies. CAFs have been shown to induce tumor growth by different experimental models, such as *in vitro*, mouse, and human patient models. One of the studies indicated the promoting effect of CAFs, as compared to normal fibroblasts, in inducing the progression of initiated non-tumorigenic prostatic hyperplasia into a tumor growth. Interestingly, under identical experimental condition, the CAFs were unable to induce the growth of normal prostate epithelial cells [198]. This suggests that CAFs do not participate in tumor initiation process, while significantly promote the progression of an early initiated growth.

Other studies revealed that CAFs boost tumor progression and development via specific secretome activity. *CXCL12* (also called *SDF1*) secreted by CAFs enhance tumor growth via interacting with *CXCR4* receptor on tumor cells; as a result exerting different signaling cascades, which enhance tumor cell proliferation and motility. This effect has been documented in different cancer models such as breast cancer [199], endometrium cancer [200], adenocarcinoma of the esophagogastric junction [201], melanoma [202, 203], and others. Apart from *CXCL12*, using a prostate cancer model, the autocrine signaling of *CXCL14* in CAFs showed to enhance tumor growth significantly [204]. The high expression of *CXCL14* was dependent on the activation of *NOS1* in the CAFs [205].

The secretion of pro-inflammatory cytokines by CAFs plays a vital role in tumor growth and progression [206, 207]. As shown in endometrium cancer, *IL-6* secreted by CAF stimulates cancer cell proliferation via *STAT3/c-MYC* signaling pathway [208]. In a melanoma model, fibroblasts lacking *PDEF* (pigment epithelium-derived factor) could induce tumor cell

growth in vitro and in vivo, as the tumor stimulatory fibroblasts exhibited a high expression level of *IL8, SERPINB2*, and hyaluronan synthase-2 [209].

Different transcription factors have been identified in CAFs as drivers for their tumor stimulatory functions. Scherz-Shouval *et al.* showed that *HSF1* (heat shock factor 1) is highly expressed in CAF isolated from breast and lung cancer patients. Interestingly, *HSF1* could directly bind to *CXCL12* and enhances its expression in CAFs, thus mediating tumor growth and progression. They also found that high HSF1 expressing CAFs was related to poor prognosis in lung and breast cancer patients [210]. *YAP1* (Yes-associated protein 1) is another example of over-activated transcription factors in CAFs, which showed to increase the ECM stiffness thus enhancing tumor cell growth and invasion [211].

On the other hand, tumor growth stimulation can be mediated through ECM remodeling and degradation by various *MMPs* secreted by CAFs. *MMP3*, which is highly expressed by activated fibroblasts, can cleave *E-cadherin* and promote tumor progression and invasion [212]. Recent study indicate that *MMP3* expression in CAFs is lower than prostate cancer cells due to inhibition of where reactive oxygen species such as hydrogen peroxide, *MMP3* expression in CAFs, but enhanced its expression in prostate cancer cells [213]. Upon tumor cell activation, stromal fibroblasts could also secret *MMP9* [214], which is essential for breast cancer cell growth via its function in disrupting tissue polarity and architecture of microenvironment [215]. Overexpression of *TIMP1* has been recorded in CAFs; such induction played a vital role in supporting prostate and colon cancer progression *in vivo* [216]. In contrast, knocking-out all four members of *TIMP* family in fibroblasts enhanced breast cancer cell motility and cancer stem cell-like properties. *TIMP* inactivation was sufficient for CAFs markers acquisition; CAFs in turn secreted exosomes enriched with *MMPs* and ECM proteins. The authors showed *ADAM10*-rich exosomes activate *RhoA* and Notch signaling in breast cancer cells, thus driving their activity and stem cell property [217]. Another study revealed that concomitant inactivation of *Notch* effector *CSL* and *p53* stimulate CAF and tumor cell expansion [218].

1.7.3 The impact of fibroblasts on cancer metastasis

Cancer associated fibroblasts are essential intermediates of secondary tumor growth at the distant site, even though; their effect on cancer cells might start at the primary site. As CAFs secret range of cytokines, chemokines and growth factor, which in turn stimulate the cancer cell invasion and metastasis. *IL6* secreted by CAFs could activate *JAK2-STAT3* pathway in gastric cancer cells and boost their migration and the ability to undergo EMT. The inhibition

of *IL6* or *JAK2-STAT3* pathway in CAFs or cancer cell, respectively, reduced the metastatic rate to the peritoneum [219]. Another gastric cancer study showed that the high SRF expression (serum deprivation factor) in fibroblasts induces cancer cell metastasis via enhancing the *CXCL12/CXCR4* signaling [220]. The *CXCL12* producing fibroblasts can enhance *CXCL6* secretion in colon cancer cells, which consequently exhibits a high invasive and metastatic property [221]. *CXCL12* secreted by CAFs may also induce EMT as shown in oral squamous cell carcinoma [222] and breast cancer studies [223].

Interestingly, Gaggioli *et al.* showed that CAFs might act as guides at the primary site to facilitate the collective cancer invasion process, where they pave the way via generating ECM tracks and enhancing the stiffness [224]. The notion of collective migration was supported through many observations, which have revealed that cancer cells could stay as a group together maintaining their *E-cadherin* expression and do not acquire EMT [225-227]. Using a zebra fish model, CAFs isolated from prostate and colorectal cancer showed to induce cancer cell metastasis at the early stage of primary cancer growth. In the circulation, most of metastatic cancer cells showed to travel in tight association with CAFs [228]. Recently, an elegant colon cancer study showed that, CAFs induce cancer cell invasion by pulling and stretching the plasma membrane. Applying such contractile forces resulted in gap formation through the basement membrane allowing the cancer cells to move and invade easily. Interestingly, the authors observed that *MMPs* were not involved, and thus concluded that the effect was independent of basement membrane degradation [229]. Another interesting study showed that *Tenascin C* and *VEGFA* secreted by *FSP1*+ve fibroblasts at the metastatic niche, enhanced cancer cell metastasis. Depletion of *FSP1*+ve fibroblasts reduced the metastatic colonization significantly, while it did not affect primary tumor growth [230].

Cancer associated fibroblasts may induce invasion and metastasis via stimulating the angiogenic switch in the TME. In a gastric cancer mouse model, stromal fibroblasts enhance angiogenesis via *VEGFA* secretion upon activation by cancer cells [231]. Using prostate cancer xenograft model, CAFs expressing connective tissue growth factor (*CTGF*) significantly increased the micro-vessel density and tumor growth activity [232].

In recent years, the roles of exosomes in cancer biology have emerged massively, where several observations have highlighted the impact of those extra cellular vesicles on cancer invasion and metastasis [233, 234]. One of the studies demonstrated the promoting effect of CAFs derived-exosomes on lung cancer cell invasion and metastasis, via stimulating PCP (Wnt-Planar cell polarity) autocrine signaling in cancer cells [235]. An esophageal cancer

study showed that, fibroblasts upon activation by tumor cells *in vitro*, secrete exosomes holding miRNA-45, which in turn induces cancer cell growth and migration. The same miRNA was detected in the serum of 39 esophageal squamous cell carcinoma patients [236].

In order to metastasize cancer cells need to colonize into a distant tissue. Therefore they prime the target tissue in advance, and recruit stromal cells at the metastatic site [43, 237]. Malanchi *et al.* showed that infiltrating mammary cancer stem cells, could prime and recruit lung fibroblasts to overexpress periostin, which stimulate *Wnt* signaling in the cancer cells and promote their colonization efficiency [238]. Similar observations were obtained in PDAC metastasized to liver, whereas hepatic stellate (fibroblasts) cells activation and periostin induction was triggered through granulin secreted by tumor associated macrophages [239].

It is evident that CAFs are important for tumor growth, invasion and metastasis, however several observations suggested additional impact of CAFs as immune modulation and drug resistance intermediation (Figure 6).

1.7.4 CAFs as immune modulators

Cancer associated fibroblasts persistently receive-respond to the stimulations, and their secretome dynamically evolves during all tumorigenesis stages. Therefore, they potentially affect the other cells in the TME, in particular the immune cells. Most of the available evidences state CAFs as immunosuppressive agents, however the majority of observations, are based on in vitro studies. Whereas, demonstrating *in vivo* studies could be quite difficult, due to the complexity and plasticity in the TME, which keeps all different cellular and non-cellular compartments enthusiastically, interacted.

It has been detected that *CXCL12* and *CCL2* producing CAFs could recruit macrophage into the TME, and support their differentiation into TAM-2 [240]. *IL6* produced by CAFs could restrict the maturation of dendritic cells and redirect monocyte toward macrophage differentiation[241, 242]. The MDSCs could also be recruited by fibroblast-secreting chemokines; MDSCs had the potency to induce angiogenesis, participate in recruitment of T-regs, and inhibit NK and T cell activity in the TME [243]. Kraman *et al.* showed that upon depletion of *FAP* in fibroblasts (using a transgenic *FAP*-ve mouse model), only 2% of injecting tumor cells (Lewis lung carcinomas cell) could develop in a tumor, and the anti-tumorigenic effect was mediated through interferon-γ and *TNFα* beside the recruitment of *CD8*+ve T cells into the TME [244].

Similarly, a murine breast cancer study showed that CAFs, via immune suppression of TME, promote tumor development and metastasis. They found that the depletion of CAFs, via targeting *FAP*+ve cells, resulted in recruitment of cytotoxic T cells, dendritic cells and decreased the recruitment of pro-tumorigenic TAM as well as reduction in angiogenic switch [245].

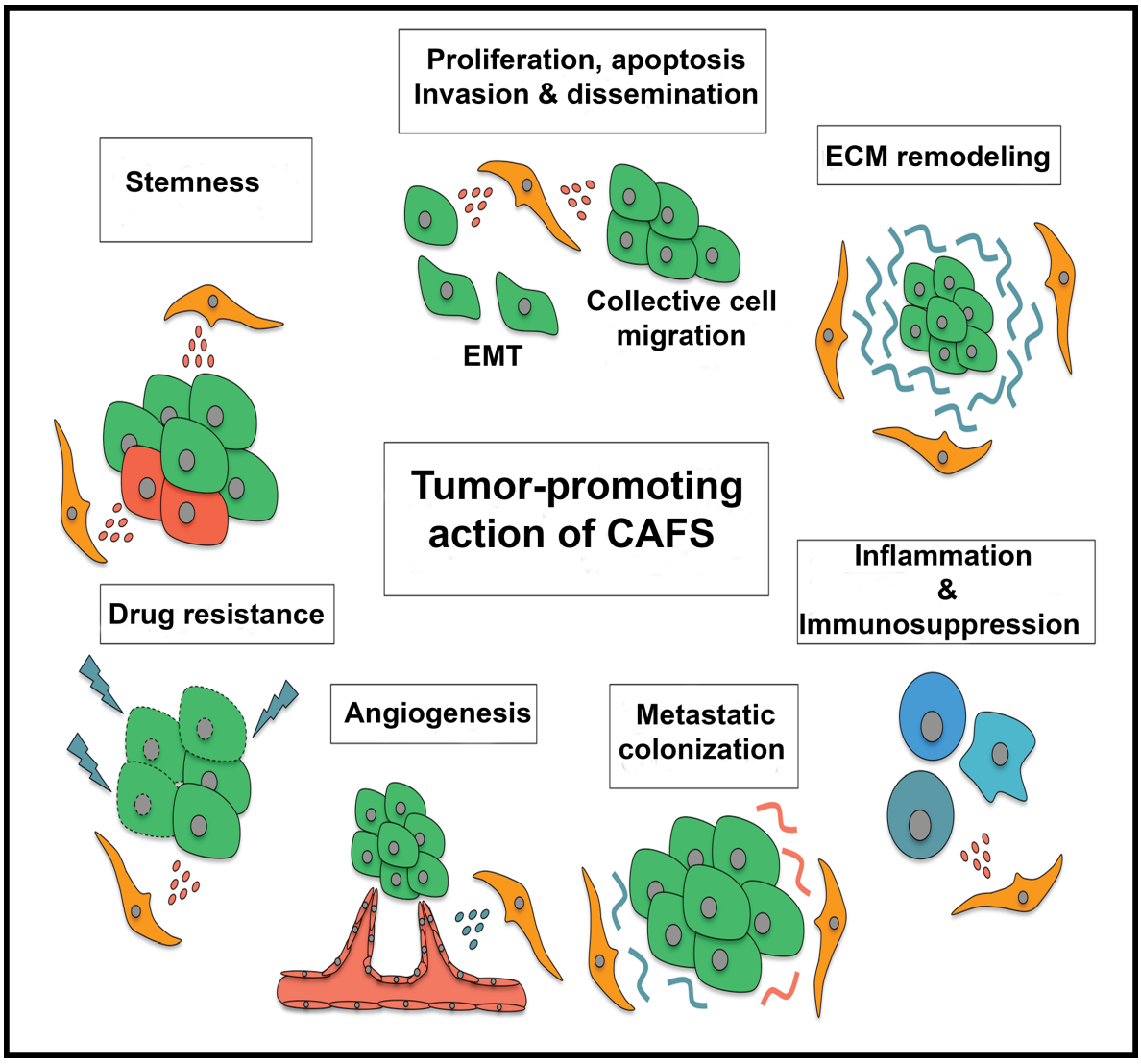

Figure 6. The role CAFs in tumorigenesis. CAFs promote tumor cell proliferation and invasion, induce stemness and drug resistance, enhance angiogenesis and ECM remodeling, facilitate metastatic colonization as well as, they increase inflammation and cause immunosuppression. Adapted and modified from [237], according to the agreement with Springer and Copyright Clearance Center.

1.7.5 CAFs and drug resistance

Beyond the availability of different treatment strategies and despite the progress made in targeting cancer, still the majority of patients get relapse or recurrence. Usually, few cancer cells or colonies sustain their survival machinery program upon the exposure to the treatment, and gradually become reprogramed. Such cells can re-grow massively while not responding

to further drug treatment; eventually, they acquire drug resistance, which is highly driven by the TME [246, 247]. Cancer associated fibroblasts, within the TME, emerged as one of the main players directing cancer cell survival and resistance to therapies. In a breast cancer study, it was proposed that elevated stromal-gene signature correlate with resistance to chemotherapy (cyclophosphamide, epirubicin and 5-fluorouracil) [248]. Cancer associated fibroblasts induce anti-cancer drug resistance through modulating the pathways involved in ECM-cancer cell interaction, cytokines and chemokines signaling, or even via CAF-cancer cell direct contact [249]. The recent *BRAF*-mutant melanoma study indicated the important role of CAFs in enhancing the resistance to *BRAF* inhibitors. A fibronectin-rich and stiffer TME was generated through the action of melanoma- associated fibroblasts; consequently, tumor cell survival was maintained by fibronectin-activating *β1-integrin-FAK-ERK* signaling in melanoma cells [250]. Another study showed that CAFs secrets MMPs that enhance anti-*EGFR* drug resistance in head and neck cancer cells [251]. Moreover, CAFs secretome conferred pro-survival signaling cascades in tumor cells upon the exposure to drug treatment. Wnt-signaling showed to be triggered in cancer cells via the secretion of *WNT16B* and SFRP2 ligand by the CAFs; *Wnt* activation attenuated the effect of cytotoxic drug on prostate cancer cells, *in vitro* and *in vivo* [252, 253]. *IL6, IL1β and IL8* showed to be overexpressed by prostate fibroblasts upon the exposure to chemotherapy, and such induction increased the rate of tumor cell survival, growth and invasion [254]. In luminal breast cancer model, the IL6 secreting CAFs augmented cancer cell survival and the resistance to tamoxifen treatment [255]. The effect of CAFs, inducing resistance to treatment, is not restricted to cytostatic drug therapy, but also to immune therapy. In a PDAC model, *CXCL12* expressing CAFs could reduce the effect of anti-*CTLA*-4 and *PD-L1* antagonists on tumor cells. Targeting the *CXCL12*-*CXCR4* signaling pathway recruits cytotoxic T cell rapidly resulting is a potent anti-tumorigenic environment, thus diminishing the PDAC cell growth [256].

Targeting CAFs in the TME is highly recommended, and anti-stromal drugs may offer new strategies to overcome drug resistance drawback. However, more systematic and comprehensive studies are required to identify the specific targetable-signaling cascades in CAFs within a specific TME.

2 AIMS OF THE THESIS

The present thesis is aiming to investigate the roles of normal versus cancer-associated fibroblasts in the tumor microenvironment.

Specifically, the thesis addresses the following questions and tasks:

1. The elucidation of the role of normal fibroblasts in the so-called “neighbor suppression phenomenon”.
2. The identification of the main signaling pathways linked to the suppressor capacity of fibroblasts upon activation by tumor cells.
3. To investigation of the possibility of switching the phenotypic behavior of fibroblasts from cancer suppressing to cancer promoting and the other way around.
4. To development of a comprehensive live cell-imaging tool, and validating it via exploring the effects of CAFs on the growth and chemotherapeutic response of cancer cells.

3 RESULTS AND DISCUSSION

The thesis presents four projects that can be divided into three categories:

- The suppression effect of normal fibroblasts on tumor growth and development (**Paper I and II**, Figure 7 and 8).
- The link between tumor suppression and tumor promoting phenotypes in fibroblasts (**Paper III**, Figure 9).
- The promoting effect of CAFs on tumor cell growth and drug resistance (**Paper IV**).

3.1 FIBROBLASTS INDUCE TUMOR GROWTH INHIBITION: THE INVOLVEMENT OF VARIOUS GENES, PROTEINS AND SIGNALING PATHWAYS

3.1.1 Neighbor suppression: the two step phenomenon

Several studies have documented the so-called neighbor suppression phenomenon. However, only few of them have demonstrated the effect-driving factors; if so they exhibited contradictory findings [145, 149, 151, 257]. Therefore, we have studied and identified the main players that direct the tumor-inhibition process by fibroblasts.

Previously, two sub-clones of the immortalized human fore skin fibroblasts BjhTERT were identified based on their differential tumor-inhibition efficiency *in vitro* [155]. The most suppressive one, also called whirly, was selected and used in our study. We have challenged the concept of neighbor suppression to prove the contact dependent assumption. To this end, we collected the secretome (the conditioned media, CM) from tumor-activated fibroblast cultures (a fibroblast-tumor cell co-culture), and from cultures of non-activated fibroblasts (fibroblasts alone), respectively. Incubating PC3 prostate cancer cells with both types of secretomes did not alter their proliferation efficiency (as measured for 5 days using a 384-well plate-high throughput inhibition assay [154]). This suggested that the tumor inhibition process couldn't be triggered by soluble factors secreted by fibroblasts. Next, we examined the contact dependent effect upon elimination of secreted, soluble factors. For this, the fibroblasts monolayer was fixed with 4% formaldehyde, keeping the ECM, the adhesive, and the trans-membrane proteins tight and intact, and PC3 cells were added. An incomplete inhibition of PC3 cell proliferation was observed. A complete inhibition was referred to the condition when PC3 cells were co-cultured with a live and dynamic fibroblast monolayer. The status of incomplete inhibition did not change when the co-culture was incubated with the CM from non-activated fibroblasts. Interestingly, the CM from the tumor-activated

fibroblasts significantly boosted the suppressive effect of the formaldehyde fixed fibroblasts monolayer; the inhibition was retained to a level close to a complete inhibition score. This in turn suggests that the activation of fibroblasts by tumor cells is crucial for stimulating the secretion of anti-tumor soluble factors, which can only be effective when there is a direct contact between fibroblasts and tumor cells. A number of studies have indicated the important role of fibroblasts in restricting tumor growth and development. Upon reducing the content of stroma, via deleting the *Sonic hedgehog* gene in a mouse PDAC, a more aggressive tumor was generated, which was characterized by an undifferentiated histology and high proliferation efficiency [258]. Using a similar PDAC mouse model, another study showed that upon targeting and depleting *αSMA*-positive fibroblasts in the mouse, a highly invasive tumor developed. Such a tumor was characterized by enhanced EMT, hypoxia, a stem cell-like phenotype, and also by reduced animal survival [259]. Interestingly, in attempting to reconstruct a human mammary epithelial tissue in mice, Weinberg and colleagues, found that normal activating fibroblasts were responsible for the configuration of normal epithelial phenotype. Furthermore, they found that patient epithelial cells injected in humanized cancer associated microenvironment yielded a cancer similar to human ductal carcinoma. Such a phenotype was normalized when the normal fibroblasts were co-injected with patient cells [260].

Furthermore, to investigate whether the neighbor suppression effect is restricted to cell proliferation or can be extended further beyond tumor cell motility, we have also monitored the motility of PC3 cells using extended field time-lapse imaging. Similarly, CM from tumor-activated fibroblasts, but not from the non-activated ones, significantly enhanced the suppression of PC3 cell motility upon contact with the formaldehyde fixed fibroblasts monolayer.

Eventually, our finding demonstrated that the neighbor suppression effect of fibroblasts against tumor cell proliferation and motility has a multifactorial effect assembled into two steps: the first representing the ECM, the adhesive and the trans-membrane proteins in fibroblasts, which via direct contacts with tumor cells initiate the process of inhibition. Such fibroblasts-tumor cell interactions enhance the secretory machinery (most likely in fibroblasts), which represent the second group of factors that further induce the suppression process.

3.1.2 Tumor suppressive fibroblasts exhibit different genes and proteins signatures

Since both types of CM exhibited diverse functional impacts on tumor cell proliferation and motility, we wanted to analyze the nature of those differences. In order to do so, we used an Antibody Array-Kit for checking the expression of soluble proteins in both types of CM. Out of 507 analyzed proteins, nine of them displayed differential expression pattern namely: *GDF15, MMP3, CXCL2, EMAP-II, Galectin-3, uPA, DKK1, Nidogen1*, and *EDA-A2*. *GDF-15* (growth differentiation factor 15) was the only one, which was missing in the non-suppressive CM but was highly overexpressed in CM from the suppressive phenotype, which might reflect the suppressive property of tumor activated CM. It has been shown that *GDF15* can inhibit MCF7 proliferation via inducing the activation of p53 and p21 [261]. In a transgenic mice model, the ubiquitous expression of *GDF15* resulted in an intestinal adenoma resistant phenotype [262]. However, other studies have presented *GDF15* as a soluble factor that exhibit opposite effects; showing tumor promoting effects as well [263]. Recently, *GDF15* was recommended to be included in routine prostate cancer screening strategies as a prognostic factor for aggressiveness [264]. Nevertheless, our analysis was limited to ≈500 proteins, still many secreted factors have not been identified, that could be responsible for the inhibition, including but not limited to, exosomes and microRNAs; such factors were investigated in separate study (not included in the thesis).

To identify the main genes involved in the process of cancer cell inhibition, the complete transcriptome of fibroblasts before and after activation by tumor cells was verified and compared. Using Affymetrix microarray-gene chips, more than 1000 differentially expressed genes (617 as overexpressed and 402 as downregulated genes) were identified in fibroblast upon activation by tumor cells. Among the proinflammatory, adhesion and ECM genes, eleven differentially expressed candidates have been selected. Eight of them were upregulated including *CXCL1, CXCL2, COL15A, ICAM1, IL-1b, IL-6, IL-8,* and *MYO10*, and three were downregulated *ADAMTS1, SPP1*and *TNFRSF11B*. This group of candidates was further verified via q-PCR analysis. The overexpression of proinflammatory genes might not represent the inhibition status of fibroblasts, due to the fact that most of these cytokines and chemokines were shown to be involved in induction of tumor growth, progression, invasiveness, and resistance to treatment [265, 266]. However, It has been shown that proinflammatory cytokines, via inhibiting *IGF-I*, could induce growth arrest in MCF7 breast cancer cells [267]. Therefore, one could argue that the effect of proinflammatory genes is context dependent; in our experimental setup there is a direct effect on tumor cells, whereas

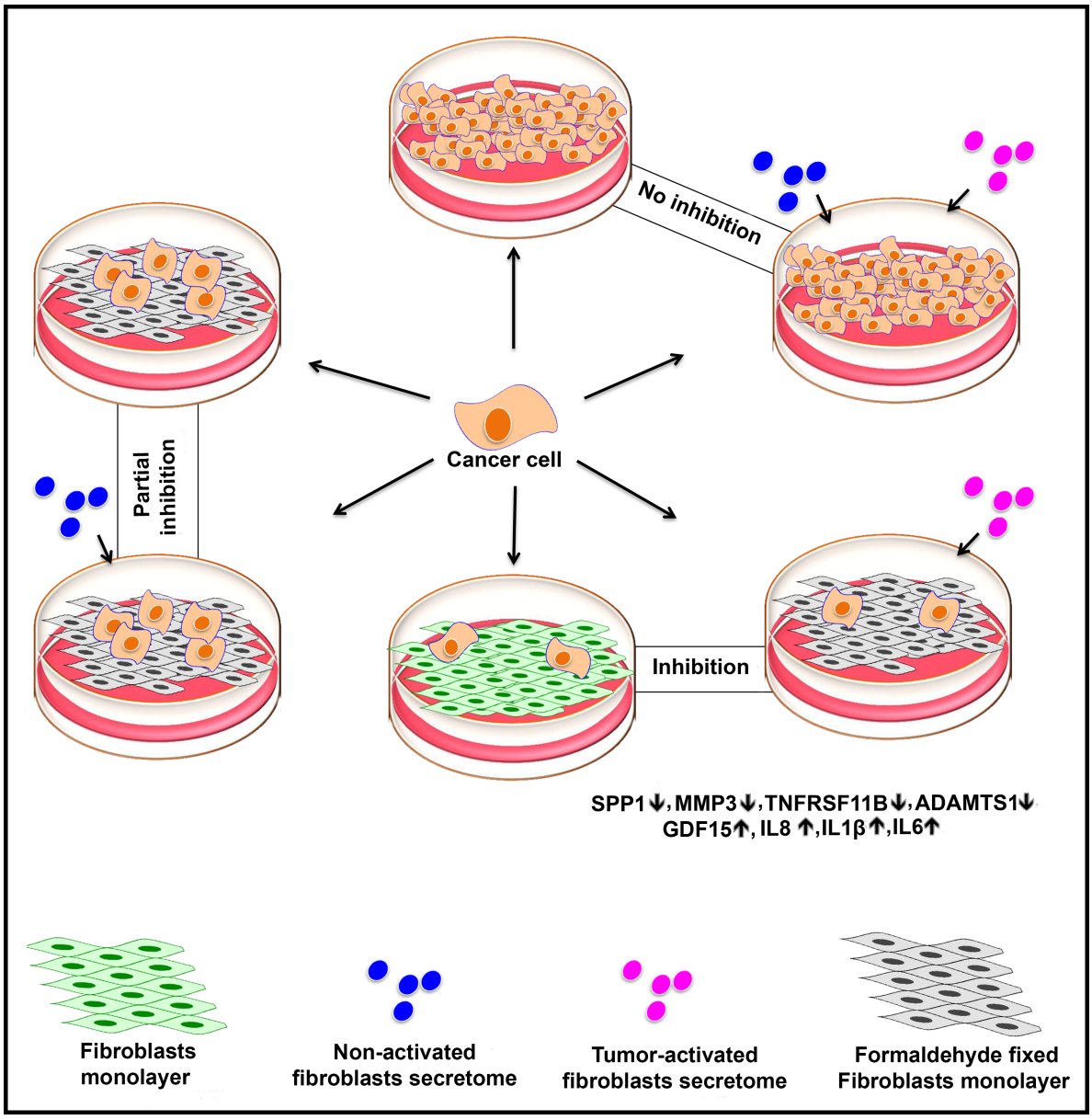

Figure 7. The inhibition of cancer cell growth is contact and soluble factor dependent.

in other models (mice and patients) the complexity of the TME, as different players interact with each other, drive the effect in different way. On the other hand, the other three-downregulated genes could be important players for controlling the inhibition of tumor cell proliferation and motility. Several observations indicated that CAFs enhance tumor growth and invasion via inducing the secretion of any of ADAMTS1, SPP1and TNFRSF11B [268-270]. The differentially expressed genes and proteins selected in our study might act collectively but not individually; the signaling cascades, triggered via the activation or suppression of selected candidates interfere with each other directing various signaling outcomes. Therefore, more comprehensive studies are required to identify the main signaling pathways involved in controlling tumor inhibition process by fibroblasts.

3.1.3 Activated- and tumor suppressive- fibroblasts exhibit differential signaling pathways signature

The notion of drawing scientific conclusions based on a differentially expressed gene or protein (cytokine, chemokine, ECM etc.), that transduces signals in one directional flow, has been argued against. Simply put, the signal transduction process represents a network of signaling pathways that operate and interact with each other to drive particular physiological processes (cell division, migration, apoptosis etc.). Applying similar concepts and using system biology approaches [271, 272], we have investigated all the possible links and pathways correlated to fibroblasts suppression effect and upon activation by tumor cells.

Four pairs of fibroblasts were used in this study; each pair represented two fibroblasts with high and low tumor suppressive functions, respectively. One of the pairs was *whirly* and *crossy*, a sub-clone of BjhTERT cell line (as mentioned previously, see page 29 and [155]), and the other three were primary cells isolated from patients and/or donors (normal and cancerous area from prostate cancer patients, adult normal skin, pediatric skin and hernial samples). All fibroblasts were co-cultured with PC3 prostate cancer cells and their inhibition score was determined after five days (using 384-well inhibition assay). The inhibition score revealed that *whirly*, normal prostate, and skin fibroblasts were more suppressive, while *crossy*, hernia, and prostate cancer fibroblasts were less or non-suppressive, respectively. At the same time, Affymetrix microarray analysis was performed on 16 samples of fibroblasts (all eight fibroblasts before and after activation by PC3 cells). Three sets of differentially expressed genes (DEG) were selected as comparing the transcriptomic signature between A) fibroblasts before and after tumor cell activation, B) suppressive *versus* non-suppressive fibroblasts before tumor cell activation, and C) suppressive *versus* non-suppressive fibroblasts after tumor cell activation. All genes that showed low expression level, as variance <0.1 and mean <4, were neglected.

To investigate the functional relevance of each DEG set, we have performed a network enrichment analysis (NEA); identifying the signaling pathways, which would be affected by a particular DEG set. The full set of DEG/group was relatively large, however, we chose and compared the significance of three sub-sets (top- 30, 100, and 300 genes that were differentially expressed) on the known signaling pathways (selected from different sources, including Reactome, KEGG, WikiPathways, BioCarta, and others). Each of these pathways composed of a set of genes/proteins, which called functional gene set (FGS); the links between DEG to FGS were determined and the observations were considered significant

when the false discovery rate (FDR) were bellow 0.1 with a minimum of 5 links. The statistical power of the observations was proportional with the size of the DEG sub-set; the 300 genes list was the most significant one and therefore was the only reported sub-set. In group A (DEG in fibroblasts after PC3 activation as compared to non-activated fibroblasts), 76 pathways were significantly enriched, however the number of links per pathway was changing between 20 to 998 links. The top five pathways that exhibited the largest number of links were: focal adhesion (998 links), *TNFα-NFκβ*-signaling pathway (932 links), regulation of actin cytoskeleton (834 links), *RhoA*-signaling pathway (824 links), and chemokine signaling pathway (770 links). Such results support previous observations, where fibroblasts upon interaction with tumor cells, showed to change their contractile and stiffness status [273, 274]. In group B (DEG in suppressive fibroblasts as compared to non-suppressive fibroblasts before PC3 activation), 56 pathways were significantly enriched. The first 5 pathways indicated with highest number of links were: *RhoA*-signaling pathway (578 links), focal adhesion (512 links), chemokine signaling pathway (476 links), *Wnt*-signaling pathway (450 links) and regulation of actin cytoskeleton (442 links). Interestingly, in group C (DEG in suppressive fibroblasts as compared to non-suppressive fibroblasts after PC3 activation), only one pathway was enriched (the *TNFα-NFκβ*-signaling pathway) but with the highest number of links (1572) as compared to all other groups. Changes in this pathway and its signaling cascades have been shown to be involved in both tumor growth as well as in regression [275].

It is important to highlight that our DEG sets were obtained via considering all 8 fibroblasts similar, however they were originating from different sites and tissues as well as being primary or immortalized cells. Therefore, we have selected two pairs (first pair: *whirly* and *crossy* that originate from skin and they are immortalized fibroblasts, and second pair: normal prostate and cancerous prostate fibroblasts, which represent primary *ex vivo* cells), to analyze whether their pathway score is correlated to each other or not. We observed that both pairs were highly associated on the pathway level (Spearman rank $R = 0.686$, $p < 10^{-18}$), despite that they showed differences on the DEG list (only 59 DEGs were identical).

Apart from the signaling pathways, and via using NEA, we have also identified a number of transcription factors and other regulatory genes, which were enriched in each set of DEGs. In this way we could identify individual genes, which were not known to be members of any particular pathway; for example, we found that *RelA* (P65), a *NFκβ*-signaling mediator, regulate genes involved in *Rho*-signaling such as *NET1* (neuroepithelial cell transforming factor), *NFKBIA* (NF-kB inhibitor), *IL1B*, the *RELT* (TNF receptor), and *BHLHE40*.

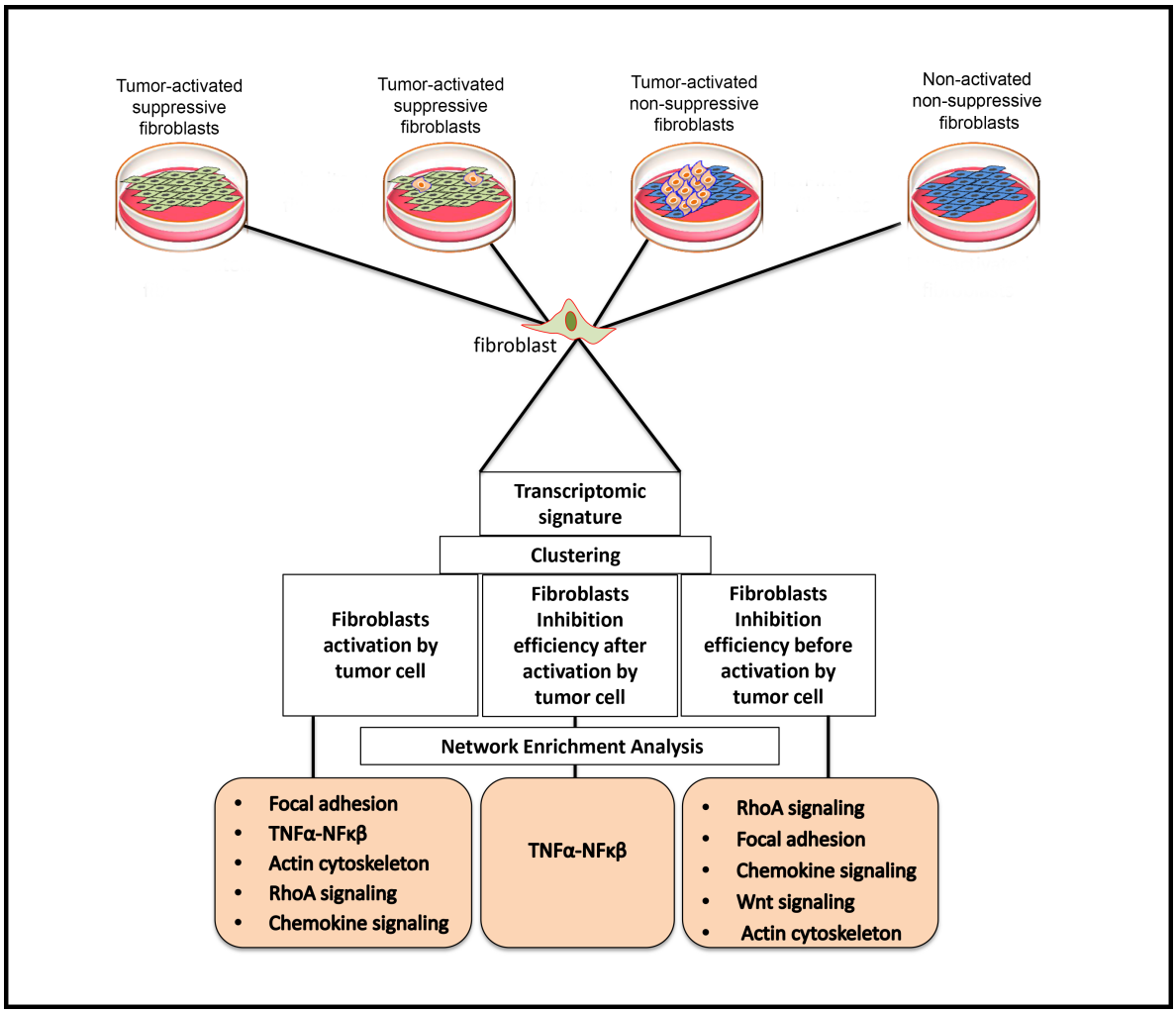

Figure 8. The potential signaling pathways altered in fibroblasts, upon activation by tumor cells and in correspondence to the tumor suppressive functions.

3.2 FIBROBLAST ACTION: FROM ANTI-TUMORIGENIC INTO PRO-TUMORIGENIC PHENOTYPE

3.2.1 Targeting *RhoA* gene in fibroblasts enhances tumor cell proliferation and motility

RhoA-signaling plays an important role in tumor growth and migration [276, 277]. Both our recent and previous data indicated the role of *RhoA*-signaling in the TME. As we showed via in NEA (mentioned in the previous section, page 33), *RhoA*-signaling was ranked the first among all the signaling pathways, which significantly correlated to fibroblasts suppression effect against tumor cell growth and proliferation. Additionally, our group have previously identified 12 novel markers for CAFs, where four of them were related to *Rho* GTPase signaling and the fifth one was related to *RhoA*-signaling. Therefore, to investigate the direct

effect of *RhoA* on the functions of fibroblasts as anti- or pro-tumorigenic cells in the TME, we have targeted and deleted the *RhoA* gene in the fibroblasts.

Using CRISPR/cas-lenti virus knocking-out system, we could eliminate the *RhoA* gene in BjhTERT fibroblasts. The expression level of *RhoA* on the gene and protein level was confirmed. The rate of tumor cell (PC3 cells) proliferation was determined in the presence of *RhoA*-knock out BjhTERT cells and their corresponding control cells (infected with empty vector-lenti viral particle). Tumor cell proliferation efficiency was increased significantly in the presence of *RhoA* deficient fibroblasts as compared to *RhoA* expressing fibroblasts.

We also have examined the effect of *RhoA* expression on the function of fibroblasts as stimulator or suppressor of tumor cell motility in vitro. The motility of PC3 cells was increased significantly (they also exhibited big sized colonies) upon co-culture with *RhoA* deficient BjhTERT as compared to the co-culture with *RhoA* expressing BjhTERT cells (control cells). Our results indicate that the suppressive effect of fibroblasts against tumor cell proliferation and motility can be modulated into a stimulatory one, with *RhoA* as the suggested main driver of this modulation.

3.2.2 *RhoA* knock-out fibroblasts induce tumor formation in mice and tumor cells compactness in 3D-collagen matrix

To investigate the effect of *RhoA* deficient fibroblasts on tumor initiation and development *in vivo*, we have co-injected PC3 and BjhTERT (*RhoA* knock-out and wt control cells) fibroblasts in SCID and SCID-beige mice. Our experimental setup included the injection of a non-tumorigenic dose of PC3 cells; 20.000 PC3 cells when injected alone were unable to initiate tumor growth *in vivo*. Upon co-injection of the same number of PC3 cells with 1 x 10^6 BjhTERT control cells, only two out of fifteen mice developed small tumors. Whereas all mice co-injected with the PC3-*RhoA* deficient fibroblasts got tumors; they started to grow after 6 – 7 weeks, followed by an enormous growth where the size of tumor mass became 1 cm^3 in two weeks.

Our *in vivo* and *in vitro* observations highlight the importance of *RhoA* expression in fibroblasts to maintain their anti-tumorigenic phenotype. The inactivation of *RhoA* gene expression in fibroblasts or TME showed a pro-tumorigenic action. On the contrary, activation of *RhoA*-signaling in cancer cells appeared to be vital for their growth and invasiveness. It has been shown that activation of *RhoA* in human mammary epithelial cells enhances their pre-neoplastic transformation and directed their immortalization [278].

Another study showed that *RhoA* was mutated in more than 25% of diffuse-type gastric carcinoma; where the mutations showed a gain-of-function behavior [279].

Furthermore, we have analyzed the growth of tumor cells in 3D collagen matrix. Here PC3 cells were co-cultured with *RhoA* deficient and control BjhTERT cells, in collagen spheres for seven days, respectively. We found a significant induction of PC3 cells clustering and compactness in *RhoA* deficient fibroblasts co-cultures as compared to control fibroblasts co-cultures. Such compactness of tumor cells increases the efficiency of cell-cell contact, and may boosts cancer cell survival, as well as it may induce the stem cell like properties in PC3 cells. This finding support our *in vivo* observations, where the onset of the tumor was delayed to week six or seven, but precipitously increased within two weeks later. We have hypothesized that, six weeks were required to induce the compactness and stemness in cancer cells, and when such level of compactness was achieved the cancer stem cells boosted the growth massively. It has been shown that cancer stem cell-phenotype could be enhanced by cell-cell contact and via soluble factor secreted by the TME [280]. Therefore, we wanted to investigate what kind of secretory function the BjhTERT- *RhoA* deficient fibroblasts display when they are in co-culture with tumor cells. We therefore performed an Affymetrix microarray analysis on both BjhTERT (control and *RhoA* deficient) cells, with and with out PC3 co-culture. Interestingly we found that BjhTERT-*RhoA* deficient fibroblasts, in response to tumor cell stimulations, overexpress high amount of proinflammatory genes including *IL8, IL-1α, IL-6, IL-1β, CCL2*, and *TNFAIP2,* which were further validated by qPCR analysis. This finding further supports our stemness induction hypothesis. Recently, it was shown that mesenchymal-stromal cell in the TME induced stemness of osteosarcoma cells via secreting *IL8, IL6, CXCL1, CXCL5, CCL5* and activating *NF-κβ* signaling [281]. In addition, another study showed that inflammation induces the expression of stemness-related markers in hepatocellular carcinoma [282].

3.2.3 *RhoA* knock-out fibroblasts exhibit differential cytoskeleton structure and stiffness properties

Upon the elimination of *RhoA* expression in BjhTERT, the fibroblasts displayed various modifications in the structure of cytoskeleton, represented mainly by less actin stress fiber and large focal adhesion. They also showed a very low αSMA-expression level, as compared to the control cells. Suggesting that such tumor stimulatory fibroblasts does not follow the classical CAFs characteristics; our results further supported other findings, which indicated that *αSMA* is not the universal marker for CAFs [283].

To evaluate whether the cytoskeleton modifications were related to the alterations in the mechanical properties of the fibroblasts, both the stiffness and contractile force were measured via atomic force and traction force microscopy, respectively. *RhoA* deficient fibroblasts showed a significant decrease in contractile force, but a more homogenous and distributed elastic modulus, as compared to control fibroblasts. The elastic modulus represents the cell stiffness, which was significantly higher in *RhoA* deficient cells. Such shift in fibroblasts phenotype, due to the knocking-out of *RhoA* gene, clearly highlights their tumor stimulatory phenotype. It has been documented that alterations in the mechanical properties of the TME induce tumor initiation and development [284]. Recently, it was shown that Biglycan induced fibroblasts stiffness, which promotes melanoma cell growth and invasiveness [285].

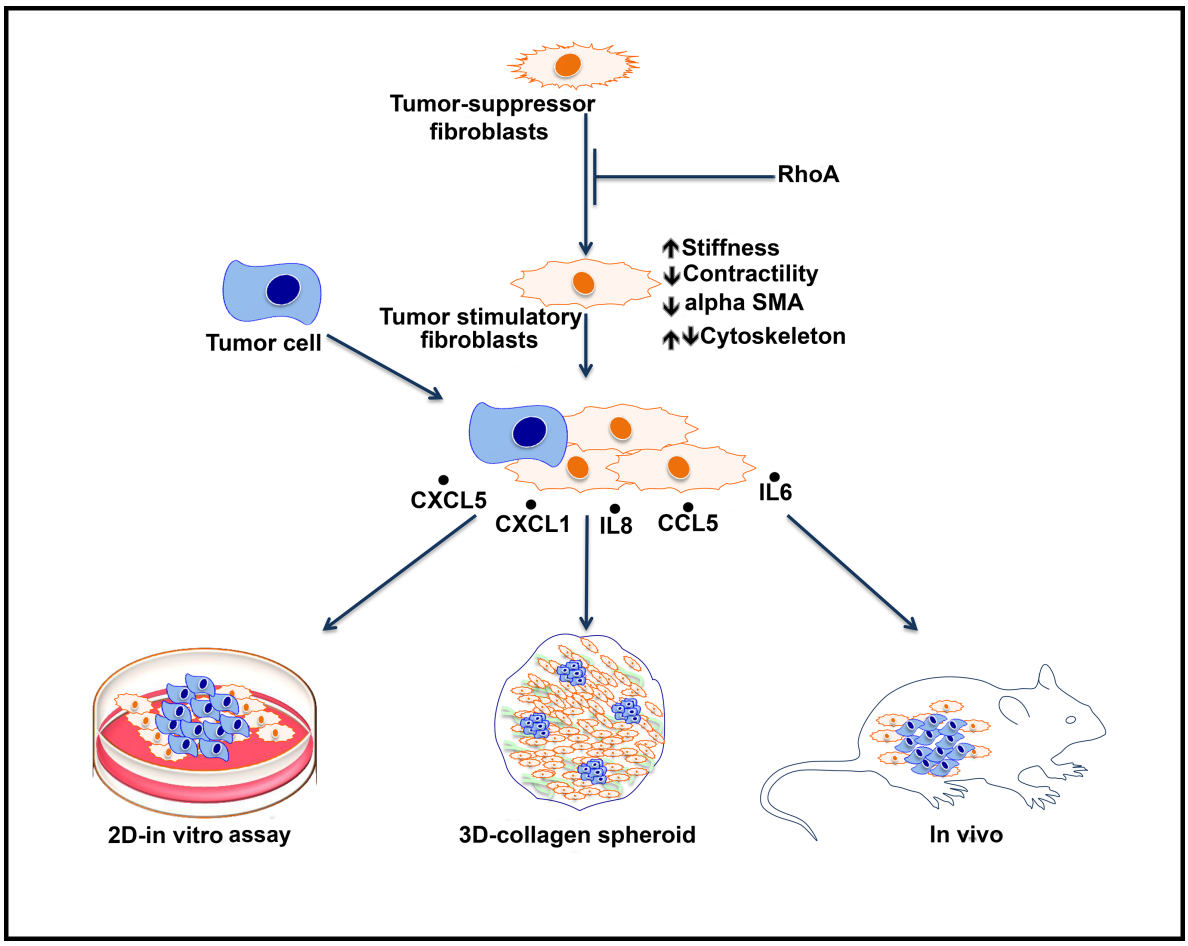

Figure 9. Tumor suppressive fibroblasts turn into tumor promoting cells. Targeting the *RhoA* gene in fibroblasts boosts tumor initiation *in vivo*, induces tumor colony compactness in 3D-collagen matrix, and reduces tumor suppressive function *in vitro*.

3.3 CANCER ASSOCIATED FIBROBLASTS MODULATE TUMOR CELL GROWTH AND THEIR RESPONSE TO CHEMOTHERAPY: METHOD DEVELOPMENT AND APPLICATION

3.3.1 Developing a quantitative live cell-imaging system to study tumor-fibroblasts interactions in vitro

It is evident that fibroblasts along with the other TME compartments play an essential role driving cancer growth, invasion and response to drug treatment. Therefore, we cannot neglect the effect of the TME while studying tumor cell growth and the response to drug treatment *in vitro*. Accordingly, we have developed a tool to study the effect of cancer cell-fibroblast (TME) interactions on tumor cell activity, growth, and the response to various types of treatment. Basically, the proliferation, motility, and phenotypic plasticity of tumor cells and fibroblasts could be observed and quantified using live cell imaging system combined with quantitative MATLAB-based analysis. In this study, the tumor cells-fibroblasts co-cultures were followed continuously for three days; thus, tumor cell proliferation and motility could be measured by a kinetic rather than an endpoint method.

The need of developing a new tool was essential due to the fact that majority of available methods and *in vitro* systems are limited to one type of cell and cannot be applied to co-culture conditions. Furthermore, the existing tools are mostly determined by measuring a single parameter, which could be one of the vital physiological processes, such as cell proliferation, cell death, metabolic activity or migration. Moreover, mentioned approaches are restricted to a specific time point conditions (endpoint assays); therefore, the outcome might not be thoroughly informative. Such as, the high probability of getting false positive or negative results, using cell proliferation assay (*e.g.* BrdU / EdU incorporation assay [286]), when investigating the response of cancer cell to a particular treatment. False results can be obtained for example if few populations of cancer cells have very low proliferation capacity accompanied by active migratory and invasive properties [287, 288]. Our method provides the possibility to overcome such challenges by following tumor growth real time at single cell level, via indexing each separately

3.3.2 Induction of ovarian cancer cell growth and the resistance to chemotherapeutic drug by CAFs

To validate our method, and as a proof of principle, we have studied the effect of CAFs on epithelial ovarian cancer cell growth and their response to cisplatin (CDDP) treatment. Beside

the high mortality rate, the choice of ovarian cancer model for this study was based on the fact that the majority of patients relapses and acquires resistance to drug treatment [289]. Therefore, we wanted to understand the nature of such resistance with particular focus on the putative involvement of CAFs.

In this work, the proliferation and motility of ovarian cancer cell line (OC) and patient-derived ovarian cells (PDOC) were monitored in the presence or absence of CAFs. Our results revealed that CAFs induced cancer cell proliferation and motility. However, the effect of CAFs on PDOCs proliferation was not clear, because most of PDOCs were slow growing cells by nature (with a doubling time > 72 hours). On the other hand, the motility of PDOCs and OCs cells was dramatically enhanced in the presence of CAFs. Without measuring the motility, we would only interpret our observations based on the proliferation rate, which might then direct us into different conclusions. Therefore, it's highly important to consider both cell proliferation and motility while investigating the growth of tumor cells in any experimental condition. Additionally, our results were in line with the majority of previous observations, where they showed that CAFs induce cancer cell growth and invasion [290, 291].

Next, we tested the effect of CDDP treatment on OCs and PDOCs before and after the co-culturing with CAFs. The cancer cells heterogeneously responded to the drug, as some of them were more sensitive than the other to CDDP treatment. Interestingly, CAFs induced the survival of cancer cells under CDDP treatment; both motility and the proliferation of OCs were induced by CAFs upon treatment. The same was true for PDOCs, where the survival index showed induced motility efficacy. As mentioned earlier, CAFs were shown to play an important role in the induction of cancer cell growth and resistance to chemotherapy. For example, in a melanoma study, fibroblasts induced cancer cell resistance to vemurafenib [292]. Similarly, in a breast cancer study, exosomes produced by fibroblasts directed cancer cell resistance to radiation and chemotherapy [293]. Here, we show that CAFs drive the resistance of ovarian cancer cells to CDDP treatment. This in turn, suggests that a new treatment strategy is required, where the combinational therapy targeting CAFs and tumor cells simultaneously is necessary.

4 CONCLUSIONS

The ambition of the present doctoral thesis was to investigate the role of fibroblasts in the process of tumorigenesis. Using *in silico*, *in vitro*, *ex vivo*, and *in vivo* experimental approaches, we could clearly demonstrate that fibroblasts have a "reciprocal action in the TME". Our observations indicated that fibroblasts could either suppress or promote tumor growth and development.

Specifically, I present the following conclusions:

I. The suppression of tumor cell proliferation and motility by fibroblasts requires two signals: the first is vital and originates from the structural configuration of the transmembrane proteins of the fibroblasts, which are in tight contact with the ECM. The second signal is dependent on the soluble factors secreted upon fibroblasts-tumor cell interactions. Specifically, the first signal is essential for initiating the process of suppression, while the latter for boosting it.

II. Fibroblasts-tumor cell interactions revealed the alteration of different signaling pathways in fibroblasts, suggesting potential mechanisms for fibroblasts suppression effect against the growth of tumor cells. For example, *RhoA*, cytoskeleton reorganization, chemokine, and *TNFα-NFκβ*-signaling pathways were found to be involved in directing fibroblasts activation and suppression phenotypes.

III. *RhoA* drives the modulation of tumor suppressor fibroblasts into a tumor stimulatory ones. Eliminating the *RhoA* gene in fibroblasts resulted in: A) Inhibition of the fibroblast-mediated suppression of tumor cell proliferation and motility *in vitro*, B) Enhancement of fibroblasts-mediating tumor cell compactness in 3D-collagen spheroids, and C) Fibroblasts-mediated induction of tumor growth and development *in vivo*. Ablating of *RhoA* in fibroblast was resulted in increased cellular stiffness, disorganized cytoskeleton, and decreased cellular contractility; accompanied by induction of proinflammatory signature, only after contact with tumor cells.

IV. Our quantitative live cell-imaging tool highlighted the significance of monitoring tumor-fibroblasts interactions kinetically; implicating tumor cell proliferation and motility as essential parameters.

V. Cancer associated fibroblasts enhanced ovarian cancer cell survival and their resistance to CDDP treatment.

5 ONGOING AND FUTURE PERSPECTIVES

In **Paper I**, we identified GDF15 (a member of *TGFβ* superfamily [294]) as one of the primary candidates, which may direct the suppressive function of fibroblasts against proliferation and motility of tumor cells. Therefore, we targeted *GDF15* in fibroblasts via CRISPR/cas and shRNA lentiviral silencing system, respectively. Next, we realized that tumor cells could produce *GDF15* as well, thus we have targeted the gene in tumor cells, in the same way. The aim is to investigate the effect of *GDF15* on tumor cell-fibroblasts crosstalk and the suppressive action of fibroblasts.

We have also checked the polymorphism of *GDF15* gene in normal fibroblasts and CAFs. Aiming for the identification of specific isoforms that correlates to anti- and pro-tumorigenic properties of fibroblasts, as well as, to survival rates in patients with different types of epithelial tumors.

Furthermore, in **Paper I**, ECM produced by fibroblasts shown to plays a significant role in the suppression of tumor cell proliferation. Therefore, we aimed to compare the ECM signature between a range of normal fibroblasts and CAFs using RT-PCR ECM-kits. A set of candidates have been selected, and we now want to define the possible signaling pathways linked to the DEGs set, using the same approach that was implemented in (**Paper II**). Our main goal is to target three to four candidates from the same pathway at once (targeting the pathway rather than a single gene). The system has been optimized using a lentiviral-silencing approach in a 384-well plate.

In **Paper IV**, we developed a live cell-imaging tool to study the effect of CAFs on ovarian cancer growth and response to CDDP treatment. Now, the goal is to develop this tool further, by shifting it from 2D semi-throughput into a 3D high-throughput system. Additionally, identifying the mechanism(s) through which the CAFs induce ovarian cancer cell survival and resistance to platinum treatment, is one of our most important future goals.

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Huddinge 11:30, December 7th 2017

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